IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

UHLMANN *et al.*.

Appl. No. 09/500,991

Filing date: February 15, 2000

For: **Compounds Modulating Sister Chromatid Separation and Method for Identifying Same**

Confirmation No.: 3282

Art Unit: 1652

Examiner: Fronda, C.

Atty. Docket: 0652.2040000/EKS/FRC

Brief on Appeal Under 37 C.F.R. § 41.37

Mail Stop Appeal Brief - Patents

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

A Notice of Appeal from the final rejection of claims 36, 37, 40, 41, 43, 44, 46-49 and 58 was filed on March 30, 2006. Appellants hereby file this Appeal Brief, together with the required brief filing fee and any necessary extension of time fees.

It is not believed that extensions of time are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any fees required therefor (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 19-0036.

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I. Real Party In Interest

The real party in interest in this appeal is Boehringer Ingelheim GmbH.

II. Related Appeals and Interferences

There are no prior or pending appeals, interferences or judicial proceedings known to Appellant or the Appellant's legal representative which may be related to, directly affect or be directly affected by or have a bearing on the Board's decision in the present Appeal.

III. Status of Claims

Claims 1-35, 38, 39, 42, 45 and 50-57 have been canceled.

Claims 36, 37, 40, 41, 43, 44, 46-49 and 58* are rejected.

Claim 59 is objected to.

IV. Status of Amendments

No amendments have been filed subsequent to the Final Office Action dated October 5, 2005.

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* Although the Final Office Action dated October 5, 2005, and the Advisory Action dated May 18, 2006, list claim 59 as being rejected *and* objected to, the Examiner confirmed in a telephone conversation with the undersigned on May 25, 2006, that claim 59 is objected to and claim 58 is rejected.

V. Summary of Claimed Subject Matter

A. Overview of Claimed Subject Matter

Cell division involves the duplication of a cell's DNA, followed by the accurate physical segregation of the genetic material between the two resulting cells. The duplication of a cell's DNA results in the formation of "sister" chromatids. (Specification, page 2, lines 6-24). The sister chromatids remain attached to one another prior to their segregation into opposite cells. (*Id.*). The attachment between sister chromatids, known as "sister chromatid cohesion," is important for proper alignment of the chromatids prior to segregation. (Specification, page 2, line 25, through page 3, line 22). After each pair of sister chromaids is properly aligned, the attachment between sister chromatids is broken and the individual chromatids are pulled into opposite cells by the action of fibrous protein structures known as microtubules. (Specification, page 2, lines 6-24).

At the time of the present invention, it was appreciated in the art that sister chromatid cohesion depends on a multi-subunit complex known as "cohesin." (Specification, page 3, lines 4-7). In yeast cells, one of the cohesin subunits is called Scc1p. (*Id.*). The human homolog of yeast Scc1p is SCC1. (Specification, page 11, line 30, through page 12, line 9). It was also appreciated in the art that sister chromatid separation is dependent on a specialized sister separating protein which in humans is called "separin." (Specification, page 4, lines 3-11; page 11, line 30, through page 12, line 1). The yeast homolog of separin is Esp1p. (Specification, page 11, line 30, through page 12, line 1). Prior to the present invention, however, a direct connection between Esp1p/separin and Scc1p/SCC1 was not known or appreciated.

Through the experiments set out in the present specification, the present inventors made the surprising discovery that Esp1p cleaves Scc1p and that this cleavage is a necessary prerequisite to sister chromatid separation. (Specification, page 9, lines 10-13; page 11, lines 10-13; page 16, lines 29-32). In other words, it was discovered that sister chromatid separation, which is an essential event of cell division, involves the proteolytic cleavage of a separin substrate (*e.g.*, Scc1p) by a separin (*e.g.*, Esp1p). (*Id.*). Based on this surprising discovery, the inventors conceived of screening methods for identifying inhibitors of sister chromatid separation. (Specification, page 4, line 29, through page 5, line 6; page 17, lines 1-5). As noted in the specification, such inhibitors are useful for, *e.g.*, inhibiting the proliferation of rapidly dividing animal cells such as tumor cells. (Specification, page 17, lines 13-18).

B. The Subject Matter Defined by Independent Claim 36 and Support in the Specification Therefor

The sole independent claim involved in this Appeal is claim 36, which is directed to a method for identifying a compound that has the activity of inhibiting sister chromatid separation in eukaryotic cells. The method comprises: (a) incubating with a test compound a separin in the presence of a separin substrate. The substrate is defined as a peptide or polypeptide comprising an amino acid sequence EXXR, wherein X is any amino acid. It is also specified in the claim that the substrate is capable of being cleaved by the separin. The method also comprises: (b) determining the inhibiting effect of the test compound on the proteolytic activity of the separin. Support for independent claim 36 can be found throughout the specification, for example, at page 15, lines 10-15, at page 17, lines 6-23, and at page 17, line 24, through page18, line 18.

VI. Grounds of Rejection to be Reviewed on Appeal

There are two separate grounds of rejection to be reviewed on Appeal:

(1) ***Rejection Under 35 U.S.C. § 112, First Paragraph -- Written Description***: Whether claims 36, 37, 40, 41, 43, 44, 46-49 and 58 contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention.

(2) ***Rejection Under 35 U.S.C. § 103***: Whether the subject matter of claims 36, 37, 40, 41, 43, 44 and 48 is obvious over Brown *et al.*, *Analyt. Biochem. 217*:139-147 (1994) (Exhibit 1) in view of Ciosk *et al.*, *Cell 93*:1067-1076 (1998) (Exhibit 2).

VII. Argument

A. The Claimed Methods are More Than Adequately Described in the Specification

1. Legal Principles Relating to the Written Description Requirement

To fulfill the written description requirement of 35 U.S.C. § 112, first paragraph, a patent specification must describe an invention in sufficient detail that one skilled in the art can clearly conclude that the inventors invented the claimed subject matter. *See Regents of the Univ. of Cal. v. Eli Lilly & Co.*, 119 F.3d 1559, 1566, 43 U.S.P.Q.2d 1398, 1404 (Fed. Cir. 1997). Stated differently, the written description requirement is satisfied when the specification "set[s] forth enough detail to allow a person of ordinary

skill in the art to understand what is claimed and to recognize that the inventor invented what is claimed." *University of Rochester v. G.D. Searle & Co.*, 358 F.3d 916, 928, 69 U.S.P.Q.2d 1886, 1896 (Fed. Cir. 2004). Moreover, an important consideration in assessing written description of a claimed invention is the knowledge of one skilled in the art. *See Bilstad v. Wakalopulos*, 386 F.3d 1116, 1126, 72 U.S.P.Q.2d 1785, 1792 (Fed. Cir. 2004).

According to the Federal Circuit, "[i]t is not necessary that every permutation within a generally operable invention be effective in order for an inventor to obtain a generic claim, provided that the effect is sufficiently demonstrated to characterize a generic invention." *Capon v.* Eshhar, 418 F.3d 1349, 1359, 76 U.S.P.Q.2d 1078, 1085 (Fed. Cir. 2005). In addition, when generic elements of a claim are so well known and thoroughly characterized in the art that their recitation alone is sufficient to convey distinguishing information regarding their identity, the written description requirement for those elements is fully satisfied. *See Amgen Inc. v. Hoechst Marion Roussel Inc.*, 65 U.S.P.Q.2d 1385, 1398 (Fed. Cir. 2003).

Finally, a description as filed is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the Examiner to rebut the presumption. *See, e.g., In re Marzocchi*, 439 F.2d 220, 224, 169 U.S.P.Q. 367, 370 (CCPA 1971). The Examiner, therefore, in making a rejection must have a reasonable basis to challenge the adequacy of the written description. The examiner has the initial burden of presenting by a preponderance of evidence why a person skilled in the art would not recognize in an Applicant's disclosure a description of the invention

defined by the claims. *See In re Wertheim*, 541 F.2d 257, 263, 191 U.S.P.Q. 90, 97 (CCPA 1976).

2. Summary of The Examiner's Basis for the Written Description Rejection

The Examiner has not argued that the claimed methods themselves are not adequately described. Rather, the written description rejection is based on the Examiner's assertion that a *particular element* used in the practice of the claimed methods is not adequately described. In particular, the Examiner has argued that the specification does not provide adequate written description for the genus of *separin substrates* defined in the claims. (*See* Office Action dated March 14, 2005, page 2, lines 19-24).

As explained in detail below, the Examiner's basis for the rejection is unsupported by the evidence of record and is contrary to the current state of the law concerning the written description requirement.

3. The Subject Matter of Claims 36, 37, 40, 41, 43, 44, 48 and 49 is Adequately Described

(a) The Specification Describes Several Exemplary Separin Substrates

Claims 36, 37, 40, 41, 43, 44, 48 and 49 are directed to methods which involve the use of a separin substrate defined as being a peptide or polypeptide comprising an amino acid sequence EXXR, wherein X is any amino acid, and wherein the substrate is capable of being cleaved by a separin.

A person of ordinary skill in the art would conclude from the present specification that the present inventors invented the claimed methods involving the use of *any* separin substrate, including *any* separin substrate comprising the amino acid sequence EXXR that is capable of being cleaved by a separin. For instance, the specification states:

> In a first aspect, the invention relates to a method for identifying compounds that have the ability of modulating sister chromatid separation in plant or animal cells, characterized in that a protease with separin-like cysteine endopeptidase activity is incubated, in the presence of the substrate(s) for its proteolytic activity and optionally its co-factor(s), with test compounds and that the modulating effect of the test compounds on the proteolytic activity of the cysteine endopeptidase is determined.

(Specification, page 17, lines 6-12). There is no indication from this passage that the inventors in any way intended to limit the kinds of separin substrates that can be used in the practice of the claimed methods.

The specification also sets forth numerous exemplary separin substrates that can be used in the practice of the claimed methods, including:

- Yeast Scc1p, (Specification, page 4, lines 3-5);
- N- and C-terminally tagged variants of yeast Scc1p containing either HA or Myc epitopes, (Specification, page 6, lines 3-16 and page 28, lines 4-12);
- HA-tagged variants of yeast Scc1p containing either an R268D mutation or an R180D mutation (the *single* mutations were shown to be cleaved by separin), (Specification, page 7, lines 19-31 and page 8, lines 5-21);

- An Scc1p variant containing the FLAG epitope, the yeast VMA intein, and a chitin binding domain, (Specification, page 31, lines 1-14);
- Phosphorylated and unphosphorylated variants of Scc1p, (Specification, page 10, lines 7-10);
- Rec8p, an Scc1p homolog that replaces Scc1p in the cohesin complex of meiotic cells and that was also shown to be cleaved by separin, (Specification, page 11, lines 14-29);
- Human SCC1, (Specification, page 13, lines 8-25);
- A Myc-tagged variant of human SCC1, (Specification, page 14, line 14, through page 15, line 2);
- N- and C-terminal deletion variants of human SCC1 (used to identify the sequence EXXR as the cleavage site), (Specification, page 15, lines 3-15); and
- Rad21, an *S. pombe* homolog of Scc1p, (Specification, page 18, lines 10-13).

The cleavage of many of these substrates by separin is illustrated in working Examples in the specification. (*See* Examples 2 and 3, illustrating the use of yeast Scc1p tagged with HA epitopes; Example 5, illustrating the use of purified yeast Scc1p; Example 9, illustrating the use of untagged human SCC1; and Examples 10-13, illustrating the use of human SCC1 tagged with Myc epitopes).

The Examiner has not explained why the aforementioned exemplary separin substrates and working examples are deemed insufficient to adequately describe the genus of separin substrates encompassed by the present claims.

(b) The Specification Describes Methods for Identifying Additional Separin Substrates

Not only does the specification set forth numerous exemplary separin substrates for use in the practice of the claimed methods, but it also describes methods for generating a multitude of separin substrates. For instance, the specification notes that the *S. pombe* protein Rad21, and Rad21 derived sequences, can be used as separin substrates. (Specification, page 18, lines 10-13, "the S. pombe homologue of Scc1 (called Rad21) contains two sequences which are similar to the two known cleavage sites in Scc1, and Rad21 derived sequences may therefore be used to generate a substrate for S. pombe Esp1 (called Cut1).") The specification additionally describes various methods that can be employed to identify a wide variety of separin substrates for use in the practice of the claimed methods. For example, it is noted in the specification that:

> Based on information about the sequence specificity of the separin proteolytic cleavage site in yeast and in man, *other potential substrates for the protease can be found in other organisms*, including humans, which also allows for the design of peptides derived from these substrates, which are useful as substrates in the screening assay of the invention.

(Specification, page 19, lines 21-25, emphasis added). The specification further sets out specific exemplary methods that can be used to identify variants of known separin substrates and additional substrates. According to the specification:

> In a preferred embodiment, the substrate is a peptide containing the cleavage site of the naturally occurring substrate. The sequence specificity of the proteolytic cleavage can be determined by testing a variety of different peptides. The peptide may be of natural origin, i.e. derived from the natural SCC1, or a variant. An example for a natural peptide [is] the human SCC1 peptide as set forth [in] SEQ ID NO:1, or a fragment thereof that contains the

> separin cleavage site. Variants can be generated either by synthesising variant peptides or by mutating DNA sequence from genes encoding cohesion proteins. More specifically, other substrates for separin can be identified by searching for small DNA fragments from the yeast genome or an oligonucleotide library that can replace the normal Scc1 cleavage sites. Oligonucleotides may be inserted into a SCC1 gene (lacking both natural cleavage sites) under control of the GAL promoter on centromeric pla[s]mid. Yeast cells may be transformed with a library of such constructs and only plasmids whose modified Scc1 protein can be cleaved by the separin activity will permit growth in the presence of galactose. The peptides encoded by the positive constructs are useful as substrates for separin in the screening assay of the invention.

(Specification, page 19, line 26, through page 20, line 12). This method would allow persons of ordinary skill in the art to identify a multitude of separin substrates that can be used in the practice of the claimed methods. *The Examiner has not presented any evidence or argument to refute these contentions.*

(c) The Level of Skill In the Art Relating to the Production and Use of Proteolytic Substrates Was Extremely High

When assessing the adequacy of written description provided for a particular claimed invention, it is necessary to consider the level of skill in the art. As articulated recently by the Federal Circuit:

> The descriptive text needed to meet these requirements varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence. The law must be applied to each invention that enters the patent process, for each patented advance is novel in relation to the state of the science. Since the law is applied to each invention in view of the state of relevant knowledge, its application will vary with differences in the state of knowledge in the field and differences in the predictability of the science.

Capon, 418 F.3d at 1357, 76 U.S.P.Q.2d at 1084. Here, the level of skill and knowledge in the art relating to the production and use of proteolytic substrates was extremely high at the time of the effective filing date of the present application. For example, having knowledge of a particular cleavage motif (such as EXXR), a skilled person would simply identify and/or isolate naturally occurring peptides or polypeptides having the cleavage motif, or alternatively, produce synthetic peptides that contain the motif. The isolation of naturally occurring peptides or polypeptides having a particular cleavage motif could have easily been accomplished using, *e.g.*, genetic screening methods. (*See*, *e.g.*, Specification, page 20, lines 1-12, describing exemplary screening methods that could have easily been used to identify additional separin substrates and variants thereof).

The level of skill in the art of producing synthetic proteolytic substrates containing a particular cleavage motif was likewise extremely high. For example, at the time of the effective filing date of the present application, techniques for producing *thousands* of peptides having predetermined amino acid sequences were routine in the art. Such techniques include, *e.g.*, the production of recombinant nucleic acid molecules that encode peptides containing a protease cleavage motif, and the direct production of multiple peptides that include the cleavage motif. (*See*, *e.g.*, Rodda, "Synthesis of Multiple Peptides on Plastic Pins," in *Current Protocols in Protein Science*, John Wiley & Sons, Inc. (1997), Exhibit 3). As noted by Rodda, "The key to preparing large numbers (hundreds to thousands) of synthetic peptides in a short time and at minimal cost is to use a parallel synthesis technique which is efficient and can be done on a small scale." (*See id.*, page 18.2.1, first paragraph). Thus, the level of skill in the art of

producing multiple peptides and polypeptide sequences, including those containing a particular proteolytic cleavage motif such as EXXR, was very high.

Importantly, the Examiner has not presented any evidence or argument to suggest that the level of skill and knowledge in the relevant art was not high. In fact, from a review of the record, it appears that the Examiner has not considered the level of skill and knowledge in the art at all in the context of analyzing the written description requirement. For this reason alone -- in view of the Federal Circuit's admonition in *Capon* that the state of the art must be considered in assessing adequacy of written description -- the present rejection cannot stand.

(d) The Term "Separin Substrate" Alone Readily Conveys Distinguishing Information Concerning the Substrates

Appellants further note that the Examiner's analysis of the written description requirement is flawed because it focuses improperly on elements of the claims that are not the point of novelty of the invention. Several recent cases from the Federal Circuit confirm that, for generic elements of a claim that are well known in the art and are not themselves the point of novelty of a claimed invention, the written description requirement may be satisfied with respect to those elements by their recitation alone.

For example, in *Amgen*, the Federal Circuit held that a patent specification that disclosed only two species of vertebrate or mammalian cells nonetheless provided adequate written description support for method claims that involved the use of vertebrate or mammalian cells, generally. *See Id.*, 314 F.3d at 1332, 65 U.S.P.Q.2d at 1398. According to the court:

> the claim terms at issue here are not new or unknown biological materials that ordinary skilled artisans would easily miscomprehend. Instead, the claims of Amgen's patents refer to types of cells that can be used to produce recombinant human EPO...*the words "vertebrate" and "mammalian" readily "convey[] distinguishing information concerning [their] identity" such that one of ordinary skill in the art could "visualize or recognize the identity of the members of the genus."* Indeed, the district court's reasoned conclusion that the specification's description of producing the claimed EPO in two species of vertebrate or mammalian cells adequately supports claims covering EPO made using the genus of vertebrate or mammalian cells, renders *Eli Lilly* listless in this case.

Id., 314 F.3d at 1332, 65 U.S.P.Q.2d at 1398. (internal citations omitted, emphasis added). The court's decision was based on two principle factors:

1. That the claim terms at issue ("vertebrate" and "mammalian") did *not* refer to new or unknown biological materials that ordinary skilled artisans would easily miscomprehend; and

2. That the words "vertebrate" and "mammalian," as used in the claims, readily conveyed distinguishing information concerning their identity such that one of ordinary skill in the art could visualize or recognize the identity of members of the genus.

When the reasoning of *Amgen* is applied in the context of the present claims, it is clear that the written description requirement is more than adequately satisfied for separin substrates comprising an amino acid sequence EXXR that are capable of being cleaved by a separin.

First, the term "separin substrate" like the terms "vertebrate" and "mammalian," does not refer to new or unknown biological materials that ordinary skilled artisans would easily miscomprehend. As noted in the specification, both separins and cohesins having homology to yeast Scc1p (*i.e.*, separin substrates) were known in the art in several species. For example, as noted in the specification:

> The sequences of human homologs of budding yeast Esp1, Pds1 and Scc1 already exist in public databases. The human homologs of Esp1 and Pds1 are referred to as separin (Nagase et al., [*DNA Res. 3*:17-24] 1996; protein sequence: NCBI Acc. No. BAA11482; DNA sequence: NCBI Acc. No. D79987) and securin (Zou et al., [*Science 285*:418-422] 1999, Dominguez et al., [*Oncogene 17*:2187-2193] 1998) respectively, and the human homolog of Scc1 as SCC1 (McKay et al., [*Genomics 36*:305-315] 1996; DNA sequence: NCBI Acc. No. X98294; protein sequence: NCBI Acc. No. CAA 66940).

(Specification, page 11, line 30, through page 12, line 5). It is also noted that the sequence motif EXXR is "conserved in many SCC1 homologs in different species." (Specification, page 15, lines 10-13). Thus, the molecules identified as separin substrates in the present application were not new or unknown biological materials (even though their role as proteolytic substrates of separin for sister chromatid separation was not appreciated prior to the present invention).

Second, the claim term itself readily conveys distinguishing information concerning the identity of the substrates so that persons of ordinary skill in the art could recognize the identities of members of the genus. Persons of ordinary skill in the art would readily understand from the claim terms alone that the substrates used in the practice of the claimed methods (a) include the EXXR amino acid motif, and (b) are capable of being cleaved by separin. Thus, a skilled person would be able to readily

distinguish the separin substrates used in the practice of the claimed methods from peptides that fall outside the scope of the claim language (*i.e.*, peptides that lack the EXXR amino acid motif and/or are not capable of being cleaved by separin).

In summary, under *Amgen*, the expression "separin substrate . . . comprising an amino acid sequence EXXR, wherein X is any amino acid, and the substrate is capable of being cleaved by the separin," by itself, conveys sufficient identifying information regarding the substrates so that a person of ordinary skill in the art could visualize and/or recognize members of the genus.

The general approach to the written description inquiry set forth in *Amgen* has recently been confirmed by the Federal Circuit. For instance, in *Capon*, the Federal Circuit held that, in the context of claims to chimeric genes comprising *known* genetic elements, it was unnecessary for the applicants/patentees to provide a structural description (*i.e.*, a recitation of the nucleotide sequence) of the claimed chimeric genes. *See id.*, 418 F.3d at 1358, 76 U.S.P.Q.2d at 1084-85. According to the court, "[w]hen the prior art includes the nucleotide information [of the component DNAs], precedent does not set a *per se* rule that the information must be determined afresh." *Id.* This rationale was further endorsed in the recent case of *Falkner v. Inglis*, 448 F.3d 1357, 2006 U.S. App. LEXIS 13127 (Fed. Cir. May 26, 2006) ("it is the binding precedent of this court that *Eli Lilly* does *not* set forth a *per se* rule that whenever a claim limitation is directed to a macromolecular sequence, the specification must always recite the gene or sequence, regardless of whether it is known in the prior art.") Applied to the circumstances of the present appeal, *Capon* and *Falkner* strongly support Appellants' position that the genus of separin substrates recited in the claims is more than adequately described by virtue of

the fact that the molecules identified as separin substrates in the present application were known in the prior art.

(e) Example 18 of the USPTO's Written Description Guidelines Indicates that The Genus of Separin Substrates Defined in the Claims is Adequately Described

Example 18 of the USPTO's "Synopsis of Application of Written Description Guidelines" (available at http://www.uspto.gov/web/ menu/written.pdf, copy submitted herewith as Exhibit 4) provides further support for Appellants' position. This Example illustrates an analysis of the written description provided for a process claim where the novelty is in the method steps. The claim at issue in this Example is as follows:

> A method of producing a protein of interest comprising;
>
> obtaining *Neurospora crassa* mitochondria,
>
> transforming said mitochondria with a expression vector comprising a nucleic acid that encodes said protein of interest,
>
> expressing said protein in said mitochondria, and
>
> recovering said protein of interest.

The specification in Example 18 shows actual reduction to practice of a single embodiment: the expression of β-galactosidase. The claimed process, however, involves the use of *any* nucleic acid that encodes *any* protein of interest, a virtually unlimited genus. Nonetheless, the Example concludes that the claimed invention is adequately described. According to the analysis provided in this Example:

> The art indicates that there is no substantial variation within the genus because there are a limited number of ways to practice the process steps of the claimed invention.
>
> The single embodiment is representative of the genus based on the disclosure of *Neurospora crassa*

> mitochondria as a gene expression system, considered along with the level of skill and knowledge in the gene expression art. One of skill in the art would recognize that applicant was in possession of all of the various expression methods necessary to practice the claimed invention.

Significantly, in assessing the written description of this hypothetical claim, the USPTO's Example does not even question whether the specification provides adequate description of the entire genus of nucleic acid encoding a protein of interest because the nucleic acid is *not itself being claimed.* Thus, the emphasis in this analysis is on whether the *process* is adequately described. Structural information regarding the individual *elements* used in carrying out the process is clearly *not* required to satisfy the requirements of § 112, first paragraph under these circumstances.

Analogously, separin substrates comprising the amino acid sequence EXXR in the present claims are not themselves being claimed; they are simply elements used in the practice of the claimed methods. Thus, the written description analysis should focus on whether or not the *methods* are adequately described, not whether separin substrates comprising the amino acid sequence EXXR are adequately described (although they certainly are adequately described by, *inter alia*, the numerous exemplary substrates described in the specification in view of the advanced state of the art. *See* Sections VII.A.3(a)-(c), above).

The Examiner has summarily dismissed Appellants' analysis of Example 18 as it applies to the present claims. According to the Examiner, this Example:

> "is not directed to an assay method using a genus of substrates comprising the amino acid sequence EXXR. Example 18 is only directed toward a method of expressing a protein of interest in Neurospora crassa mitochondria."

(Advisory Action dated May 18, 2006, Continuation Sheet, lines 19-21). The Examples from the USPTO's Guidelines, however, are intended to illustrate *general principles* to be applied in a *wide range of circumstances*. Clearly, it was not the intent of the drafters of these Examples to have them apply only to the narrow circumstances set forth in these hypothetical scenarios. It is rather disturbing that the Examiner, in assessing the adequacy of written description provided for the present claims, has refused to consider the guidance provided by the USPTO's own training materials simply because the facts and circumstances of Example 18 do not precisely match the facts and circumstances surrounding the present claims.

As explained above, one of the principles taught in Example 18 is that the written description inquiry should focus on the novel aspects of the invention (*e.g.*, the process steps of the claimed method) rather than on elements of the claims that do not represent a point of novelty of the invention (*e.g.*, a nucleic acid that encodes a protein of interest). This general principle, when applied to the present claims, strongly supports Appellants' position that the subject matter of the present claims is more than adequately described. It is unfortunate that the Examiner has refused to even consider the potential applicability of the principles taught in the USPTO's Written Description Guidelines relative to the present claims.

(f) The Examiner Has Not Set Forth A Reasonable Basis for Challenging the Adequacy of Written Description for the Recited Genus of Separin Substrates

As noted above, the Examiner has the initial burden of establishing a reasonable basis for challenging the adequacy of the written description for a claimed invention. *See Wertheim*, 541 F.2d at 263, 191 U.S.P.Q. at 97. Here, the Examiner has merely

presented conclusory arguments that are unsupported by the evidence of record and, in any event, fail to take into account the current state of the law regarding written description. Thus, the Examiner's burden has not been met.

The Examiner provided the following explanation for the rejection:

> [T]he specification does not describe a substantial portion of an amino acid sequence that is common to all members of the genus of separin substrates. Thus, the skilled artisan cannot predict the structure of other species encompassed by the genus separin substrates, fragments, and variants thereof.

(Office Action dated October 5, 2005, page 3, lines 11-15). Similar arguments were asserted in the Advisory Action issued on May 18, 2006, where the Examiner stated that:

> The specification does not disclose how the length or the composition of the peptide containing EXXR would affect the ability of the separase [sic: separin] to recognize and hydrolyzes the peptide. The specification does not disclose where the EXXR should be in relation to the N- or C-terminal of the peptide which will enable the [separin] to recognize and hydrolyze the peptide. It is not clear from the specification if large peptides comprising EXXR would be hydrolyzed by [separin].

(Advisory Action dated May 18, 2006, Continuation Sheet, lines 8-11). These conclusory arguments, however, fall far short of satisfying the Examiner's initial burden of establishing a rejection for lack of adequate written description under § 112, first paragraph.

First, the Examiner's arguments ignore the fact that the specification describes multiple exemplary separin substrates from various species, including mutants and fusion proteins thereof. (*See* Section VII.A.3(a), above). Several of these exemplary substrates

are explicitly shown to function as separin substrates in working examples in the specification. (*See id.*). The Examiner has not provided any explanation or evidence to suggest that these exemplary substrates are not representative of the genus of separin substrates defined in the claims.

Second, the Examiner in making these statements, has failed to take into account the methods taught in the specification for identifying additional separin substrates. (*See* Section VII.A.3(b), above). The Examiner has also ignored the advanced state of the art and the ability of persons of ordinary skill to make, test and use a wide range of proteolytic substrates using well known and established biological techniques. (*See* Section VII.A.3(c), above). Moreover, the Examiner has not provided any explanation or evidence to suggest that a person of ordinary skill in the art, in view of these disclosed examples and the knowledge available in the art, would have been unable to recognize members of the genus of separin substrates defined in the claims.

Third, the Examiner's reasoning for the rejection, reflected in the above-quoted language, is directly refuted by recent cases and the USPTO's own Guidelines which reject the notion that structural information must be provided in order to adequately describe well known materials. As stated recently by the Federal Circuit in *Falkner*, "we hold that where, as in this case, accessible literature sources clearly provided, as of the relevant date, genes and their nucleotide sequences (here 'essential genes'), satisfaction of the written description requirement does not require either the recitation or incorporation by reference (where permitted) of such genes and sequences." *See id.*, 2006 U.S. App. LEXIS 13127 at *28. In view of the current state of the law, the Examiner's above-quoted explanations cannot support a rejection for lack of adequate written description.

Finally, with respect to the Examiner's statements regarding the length of the substrates and the location of the EXXR sequence, there is no evidence of record to suggest that determining the appropriate size of a separin substrate or the relative orientation of the EXXR sequence within the substrate would have entailed anything more than the application of routine techniques. The specification describes and illustrates through working examples several separin substrates of various lengths and compositions that are cleaved by separin. (*See, e.g.*, Section VII.A.3(a), above). In view of these examples and the disclosed methods for making and using additional separin substrates, a skilled person could have designed and implemented the full range of separin substrates defined in the present claims. Without presenting any evidence to indicate that the size of the substrate or the position of the EXXR motif within the substrate would in any way influence the ability of the substrate to be cleaved by separin, the Examiner has simply not met his burden. A rejection for lack of written description cannot be maintained on the basis of unsupported technical arguments that are contradicted by the teachings in the specification.

In view of the foregoing discussion, Appellants submit that the subject matter of claims 36, 37, 40, 41, 43, 44, 48 and 49 is more than adequately described and that a *prima facie* case of inadequate written description has not been established with respect to these claims.

4. The Subject Matter of Claims 46 and 47 is Adequately Described

Claim 46 depends from claim 36 and specifies that the substrate is human SCC1, or a fragment or variant thereof that can be cleaved by separin or having a separin cleavage site. Claim 47 depends from claim 46 and further specifies that the substrate is

a polypeptide comprising an amino acid sequence of SEQ ID NO:1, or a fragment or variant thereof that can be cleaved by separin or having a separin cleavage site. SEQ ID NO:1 is the amino acid sequence of a peptide derived from human SCC1. (Specification, page 24, lines 11-14).

Claims 46 and 47 are adequately described for at least the same reasons that claims 36, 37, 40, 41, 43, 44, 48 and 49 are adequately described. (*See* Section VII.A.3, above). In addition, the specification sets forth working examples that provide additional confirmation that the methods of claims 46 and 47 are adequately described.

For instance, Example 9 (page 38, lines 5-19 and Figs. 9A and 9B) illustrates the cleavage of SCC1 in HeLa cells following release from cell cycle arrest. Examples 10-13 (page 38, line 20, through page 40, line 16) illustrate the cleavage of a Myc epitope-tagged variant of SCC1 both *in vivo* and *in vitro* under various circumstances. The specification also teaches exemplary methods for making and using variant separin substrates. (Specification at page 20, lines 1-12, discussed in Section VII.A.3(b), above). These teachings, in combination with the working Examples, confirm that the inventors, at the time the application was filed, were in possession of the methods of claim 46 involving the use of human SCC1 and polypeptides comprising SEQ ID NO:1, as well as fragments and variants thereof.

With regard to claim 46, the Examiner stated that:

> "[t]he scope of the subgenus includes many members with widely differing structural, chemical, and physiochemical properties including widely differing amino acid sequences. Furthermore, the subgenus is highly variable because a significant number of structural differences between genus members exist."

(Office Action dated October 5, 2005, page 3, line 33, through page 4, line 1). The Examiner has failed to acknowledge that claim 46 depends from claim 36 which includes a functional limitation ("the substrate is capable of being cleaved by the separin"), and that this functional limitation significantly limits the variability among members of the genus. Additionally, the Examiner has not cited any evidence or scientific reasoning to support the assertion that the members of the genus of substrates defined in claim 46 have "widely differing structural, chemical, and physiochemical properties." Without any such evidence or reasoning, the Examiner's rejection stands on nothing more than unsupported conclusory statements which cannot support a rejection under § 112, first paragraph.

In view of the foregoing discussion, Appellants submit that the subject matter of claims 46 and 47 is more than adequately described and that a *prima facie* case of inadequate written description has not been established with respect to these claims.

5. The Subject Matter of Claim 58 is Adequately Described

Claim 58 depends from claim 36 and specifies that the substrate is human SCC1. Claim 58 is adequately described for at least the same reasons that claims 36, 37, 40, 41, 43, 44, 48 and 49 are adequately described (*see* Section VII.A.3, above), and that claims 46 and 47 are adequately described. (*See* Section VII.A.4, above). In addition, Appellants submit that none of the arguments that have thus far been advanced by the Examiner in the present record are even applicable to claim 58 because "human SCC1" is a specifically defined protein (*see, e.g.*, Specification at page 12, lines 3-5, citing the NCBI accession numbers for human SCC1 DNA and protein sequences); human SCC1 is *not* a genus of proteins.

Nonetheless, the Examiner appears to be under the impression that the term "human SCC1" refers to a genus of proteins. In the Examiner's words, "[t]he scope of the genus is not limited to . . . human SCC1 since the claims specifically recite the phrase 'comprising an amino acid sequence'." (Advisory Action dated May 18, 2006, Continuation Sheet, lines 6-7). As explained below, this interpretation of the claim scope is inaccurate as it applies to claim 58.

Independent claim 36 defines the substrate as "a peptide or polypeptide comprising an amino acid sequence EXXR . . ." Claim 58, however, depends from claim 36 and specifically defines the substrate as "human SCC1." There is no 'comprising' language in claim 58; therefore, claim 58 *is* limited to the use of SCC1. Accordingly, the Examiner's arguments regarding written description for a genus of separin substrates cannot apply to the subject matter of claim 58.

In view of the foregoing discussion, Appellants submit that the subject matter of claim 58 is more than adequately described and that a *prima facie* case of inadequate written description has not been established with respect to this claim.

B. The Claimed Methods are Not Obvious Over the Cited References

1. Legal Principles Relating to Nonobviousness

In order to establish a *prima facie* case of obviousness, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. *See In re Rouffet,* 149 F.3d 1350, 1357, 47 U.S.P.Q.2d

1453, 1457-58 (Fed. Cir. 1998). In addition, all of the claim limitations must be taught or suggested by the prior art. *See In re Royka*, 490 F.2d 981, 180 U.S.P.Q. 580 (CCPA 1974). Moreover, if an independent claim is nonobvious under 35 U.S.C. § 103, then any claim depending therefrom is likewise nonobvious. *Se In re Fine*, 837 F.2d 1071, 5 U.S.P.Q.2d 1596 (Fed. Cir. 1988).

2. Summary of The Examiner's Basis for the Obviousness Rejection

Claims 36, 37, 40, 41, 43, 44 and 48 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Brown *et al.*, *Analyt. Biochem. 217*:139-147 (1994) ("Brown") (Exhibit 1) in view of Ciosk *et al.*, *Cell 93*:1067-1076 (1998) ("Ciosk") (Exhibit 2). (Office Action dated October 5, 2005, page 4, lines 18-20). According to the Examiner:

> It would have been obvious to one of ordinary skill in the art at the time the invention was made to modify the process of Brown et al. such that the separain [sic] and yeast substrate Scc1 taught by Ciosk et al. is used in the process taught by Brown et al., where the yeast substrate Scc1 is labeled at one end with a UV/blue fluorophore and at the other end a quencher. One of ordinary skill in the art at the time the invention was made would have been motivated to do this for the purposes of having a fast and simple process for identifying separase [sic: separin] inhibitors.

(Office Action dated March 14, 2005, page 4, lines 11-16). As explained below, this justification for the rejection is factually flawed and legally insufficient to establish a *prima facie* case of obviousness.

3. Summary of Cited Art

(a) Brown

Brown refers to "a general radiometric assay for studying the proteolytic activities of endopeptidases using a tritiated-biotinylated peptide." (*See* Brown, page 145, sentence bridging left and right columns). Brown illustrates the assay using cathepsin G, a synthetic substrate, and an inhibitor known as ACT. (*See id.*, page 145, left column, lines 1-10). According to Brown:

> Although specifically used here for an enzyme which cleaves at the N-terminus of BAP, this type of labeled peptide substrate is readily applied to the detection of other endoprotease activities, as well as inhibitors of these activities.

(*See id.*, page 147, left column, lines 25-30).

(b) Ciosk

Ciosk refers to the destruction of a yeast protein called Pds1p by a ubiquitin protein ligase known as the anaphase promoting complex (APC). (*See* Ciosk, page 1067, abstract, lines 7-12). According to Ciosk, destruction of Pds1p is the APC's sole role in triggering Scc1p's dissociation from chromatids. (*See id.*). Ciosk proposes that "the APC promotes sister [chromatid] separation not by destroying cohesins but instead by liberating the 'sister-separating' Esp1 protein from its inhibitor Pds1p." (*See id.*, abstract, lines 13-15).

4. Claims 36, 37, 40, 41 and 48 are Not Obvious Over the Cited References

(a) The Cited References Do Not Teach or Suggest that Sccl p is a Proteolytic Substrate

The Examiner has relied on Ciosk for allegedly "teach[ing] a recombinant separin called Esp1p and its yeast substrate Scc1." (Office Action dated March 14, 2005, page 4, lines 9-10). There is nothing in Ciosk, however, to indicate that Scc1p is a proteolytic substrate of Esp1p. In fact, there is no suggestion whatsoever in Ciosk that Esp1p is a protease at all. Ciosk simply concludes that "Esp1p is required for both sister [chromatid] separation and dissociation of Scc1p from chromatin." (*See* Ciosk, page 1070, right column, lines 38-40). Significantly, Ciosk suggests that whatever factor causes Scc1p to dissociate from chromatin is not necessarily responsible for its destruction. According to Ciosk, "Scc1p in yeast is indeed destroyed in an APC-dependent manner, but the timing of this event suggests that *it might be a consequence rather than a cause* of its dissociation from chromosomes." (*See id.*, page 1073, right column, lines 27-30, emphasis added). Thus, the conclusion in Ciosk that Esp1p causes Scc1p to dissociate from chromosomes would not suggest that Esp1p had any role in destroying Scc1p since it is noted that the destruction of Scc1p was believed to be a consequence, *not a cause*, of its dissociation from chromatin. A person of ordinary skill in the art, based on this reference, would therefore have had no reason to believe that Esp1p was involved in the destruction of Scc1p or that Esp1p is a protease.

As noted above, there is nothing in Ciosk to suggest that Esp1p is a protease. In terms of a proposed biological role for Esp1p, Ciosk states that:

> Esp1p might, for instance, interact transiently with cohesins and facilitate their *dissociation* from chromosomes. Alternatively, it might destroy cohesion by an *indirect mechanism*, by *generating a global change within nuclei* that is more directly responsible for weakening sister chromatid cohesion. A candidate would be the concentration of Ca^{2+}, which appears to change at the metaphase to anaphase transition.

(*See* Ciosk, page 1074, left column, line 63, through right column, line 7, emphasis added, internal citation omitted). Based on Ciosk, a person of ordinary skill in the art would have had no reason to believe that Esp1p is a protease, especially since Ciosk explicitly suggests that Esp1p may function by mechanisms such as altering the nuclear concentration of Ca^{2+}, a mechanism which does not in any way suggest a proteolytic role for this gene product. The Examiner has not provided any explanation as to why a person of ordinary skill in the art would have had reason to believe that Esp1p is a protease when Ciosk explicitly suggests alternative activities for this molecule.

In summary, nothing in Ciosk would have suggested that Esp1p is a protease or that Scc1p is in any way destroyed by Esp1p. Thus, a person of ordinary skill in the art, in view of Brown and Ciosk, would have had no motivation to include Esp1p and/or Scc1p in the proteolytic assay of Brown.

(b) The Examiner Has Relied on the Teachings in Appellants' Own Specification to Justify the Rejection

The Examiner has not presented any arguments or evidence to contradict Appellants' position that Ciosk neither teaches nor suggests that Scc1p is a proteolytic substrate. Instead, the Examiner has relied on passages from *Appellants' own specification* and has presented a legally improper theory of inherency. In particular, the

Examiner has cited to Appellants' specification at page 4, lines 3-11 and at page 36, line 21 to support the assertion that Scc1p is a substrate Esp1p. (Office Action dated October 5, 2005, page 4, line 25, through page 5, line 6, "The specification states the following regarding Esp1p as taught by Ciosk et al. (1998) on page 4, lines 3-11 . . ."). According to the Examiner:

> Thus, the examiner takes the position that the Esp1p taught by Ciosk et al. *inherently* is a separin and the yeast substrate Scc1 disclosed by Ciosk et al. *inherently* is a substrate for the taught Esp1p.

See October 5, 2005 Office Action, page 5, lines 7-9, emphasis added). This reasoning is directly contrary to legal precedent and cannot stand as a basis for an obviousness rejection.

First, it is well established that, in an obviousness rejection, the teaching or suggestion to make a claimed combination must be found in the prior art, not in an Applicant's disclosure. *See In re Vaeck*, 947 F.2d 488, 20 U.S.P.Q.2d 1438 (Fed. Cir. 1991). With regard to the present invention, proteolytic cleavage of Scc1p by Esp1p is *the inventors' discovery*. Clearly, the Examiner cannot rely on passages from Appellants' own specification regarding Appellants' own scientific discovery to support a theory of motivation to combine references.

Second, it is equally well established that an obviousness rejection cannot be based on what is asserted to be *inherent* in a reference. *See In re Spormann*, 150 USPQ 449, 452 (CCPA 1966). That which is inherent cannot be obvious, since inherent information "is not necessarily known . . . [and] Obviousness cannot be predicated on what is unknown." *Id. See also In re Newell*, 891 F.2d 899, 901, 13 USPQ2d 1248,

1250 (Fed. Cir. 1989) ("a retrospective view of inherency is not a substitute for some teaching or suggestion which supports the selection and use of the various elements in the particular claimed combination.") Since proteolytic cleavage of Scc1p by Esp1p was unknown prior to Appellants' discovery and is not disclosed or suggested in Ciosk, this information cannot form the basis of an obviousness rejection. Since the courts have explicitly rejected the use of inherency as a basis for establishing obviousness, the Examiner's assertion of obviousness in the present case cannot be maintained.

In summary, the Examiner has presented no evidence outside of Appellants' own disclosure to indicate that a person of ordinary skill in the art would have believed that Scc1p is a proteolytic substrate of Esp1p or that Esp1p is a protease. Without some indication *in the prior art* that Esp1p is a protease and that Scc1p is a proteolytic substrate of Esp1p, a person of ordinary skill in the art would have had no motivation to combine Ciosk with Brown. Without a motivation to combine references, the obviousness rejection cannot be maintained.

5. Claim 43 is Not Obvious Over the Cited References

Claim 43 depends from claim 36 and specifies that the separin is human separin. Claim 43 is not obvious over the cited references for at least the same reasons that claims 36, 37, 40, 41 and 48 are not obvious over the cited references. (*See* section VII.B.4, above). In addition, neither Brown nor Ciosk teaches or suggests the use of human separin for *any purpose*. In fact, the Examiner has not addressed this element of claim 43 whatsoever. Thus, the obviousness rejection of claim 43 is legally insufficient and cannot be maintained.

6. Claim 44 is Not Obvious Over the Cited References

Claim 44 depends from claim 36 and specifies that the substrate is a protein recombinantly produced in baculovirus in the presence of a phosphatase inhibitor. Claim 44 is not obvious over the cited references for at least the same reasons that claims 36, 37, 40, 41 and 48 are not obvious over the cited references. (*See* section VII.B.4, above). In addition, neither Brown nor Ciosk teaches or suggests a protein recombinantly produced in baculovirus. Neither Brown nor Ciosk teaches or suggest the production of a recombinant protein in the presence of a phosphatase inhibitor. In fact, the Examiner has not addressed these elements of claim 44 whatsoever. Thus, the obviousness rejection of claim 44 is legally insufficient and cannot be maintained.

C. Conclusions

In view of the foregoing discussion, Appellants submit that the subject matter defined by claims 36, 37, 40, 41, 43, 44, 46-49 and 58 is more than adequately described in the specification and that the Examiner has not met his burden of establishing a *prima facie* case of inadequate written description under 35 U.S.C. § 112, first paragraph.

Appellants also submit that the subject matter of claims 36, 37, 40, 41, 43, 44 and 48 is not obvious over the cited references. The Examiner has failed to establish that all of the elements of the claims are taught or suggested by the cited references and has failed to provide evidence or a reasonable explanation as to why a person of ordinary skill in the art would have been motivated to modify or combine the references. Thus, a *prima facie* case of obviousness under 35 U.S.C. § 103 has not been established.

Accordingly, Appellants respectfully request that the Board reverse the Examiner's written description and obviousness rejections and remand this application for issue.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Frank R. Cottingham
Attorney for Appellants
Registration No. 50,437

Date: AUG. 30, 2006

1100 New York Avenue, N.W.
Washington, D.C. 20005-3934
(202) 371-2600

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VIII. Claims Appendix

36. A method for identifying a compound that has the activity of inhibiting sister chromatid separation in eukaryotic cells, said method comprising:

(a) incubating with a test compound a separin in the presence of a separin substrate, wherein said substrate is a peptide or polypeptide comprising an amino acid sequence EXXR, wherein X is any amino acid, and the substrate is capable of being cleaved by the separin; and

(b) determining the inhibiting effect of the test compound on the proteolytic activity of the separin,

wherein a compound determined in (b) to inhibit the proteolytic activity of the separin has the activity of inhibiting sister chromatid separation in eukaryotic cells.

37. The method of claim 36, wherein said eukaryotic cell is an animal cell.

40. The method of claim 36, which is high-throughput.

41. The method of claim 36, wherein said separin is recombinant.

43. The method of claim 36, wherein said separin is human separin.

44. The method of claim 36, wherein said substrate is a protein recombinantly produced in baculovirus in the presence of a phosphatase inhibitor.

46. The method of claim 36, wherein said substrate is human SCC1, or a fragment or variant thereof that can be cleaved by separin or having a separin cleavage site.

47. The method of claim 46, wherein said substrate is a polypeptide comprising an amino acid sequence of SEQ ID NO:1, or a fragment or variant thereof that can be cleaved by separin or having a separin cleavage site.

48. The method of claim 36, wherein said substrate comprises a label which generates a detectable signal proportional to the amount of the cleavage product of the proteolytic activity, and wherein the signal is measured in the presence and in the absence of the test compound.

49. The method of claim 48, wherein said label is fluorescent.

58. The method of claim 36, wherein said substrate is human SCC1.

59. The method of claim 46, wherein said substrate is a polypeptide comprising an amino acid sequence of SEQ ID NO:1.

IX. Evidence Appendix

Exhibit	Title of Exhibit	Location in Record
Exhibit 1	Brown *et al.*, *Analyt. Biochem.* *217*:139-147 (1994)	Submitted in an Information Disclosure Statement filed by Applicants on August 3, 2000, and cited by Examiner in Office Action dated March 14, 2005.
Exhibit 2	Ciosk *et al.*, *Cell 93*:1067-1076 (1998)	Submitted in an Information Disclosure Statement filed by Applicants on August 3, 2000, and cited by Examiner in Office Action dated March 14, 2005.
Exhibit 3	Rodda, "Synthesis of Multiple Peptides on Plastic Pins," in *Current Protocols in Protein Science*, John Wiley & Sons, Inc. (1997)	Submitted by Applicants with the Amendment and Reply Filed on July 14, 2005.
Exhibit 4	Example 18 of USPTO's "Synopsis of Application of Written Description Guidelines" (available at http://www.uspto.gov/ web/ menu/written.pdf)	Submitted by Applicants with the Amendment and Reply Filed on March 30, 2006.

X. Related Proceedings Appendix

No decisions have been rendered by a court or the Board in any related proceeding.

Example 18: Process claim where the novelty is in the method steps.

Specification: The specification teaches a method for producing proteins using mitochondria from the fungus *Neurospora crassa*. In the method, mitochondria are isolated from this fungus and transformed with a mitochondrial expression vector which comprises a nucleic acid encoding a protein of interest. The protein is subsequently expressed, the mitochondria is lysed, and the protein is isolated. The specification exemplifies the expression of β-galactosidase using the claimed method using a cytochrome oxidase promoter.

Claim:

1. A method of producing a protein of interest comprising;

obtaining *Neurospora crassa* mitochondria,

transforming said mitochondria with a expression vector comprising a nucleic acid that encodes said protein of interest,

expressing said protein in said mitochondria, and

recovering said protein of interest.

Analysis:

A review of the specification reveals that *Neurospora crassa* mitochondrial gene expression is essential to the function/operation of the claimed invention. A particular nucleic acid is not essential to the claimed invention.

A search of the prior art reveals that the claimed method of expression in *Neurospora crassa* is novel and unobvious.

The claim is drawn to a genus, i.e., any of a variety of methods that can be used for expressing protein in the mitochondria.

There is actual reduction to practice of a single embodiment, i.e., the expression of β-galactosidase.

The art indicates that there is no substantial variation within the genus because there are a limited number of ways to practice the process steps of the claimed invention.

The single embodiment is representative of the genus based on the disclosure of *Neurospora crassa* mitochondria as a gene expression system, considered along with the level of skill and knowledge in the gene expression art. One of skill in the art would recognize that applicant was in possession of all of the various expression methods necessary to practice the claimed invention.

Conclusion:

The claimed invention is adequately described.

ANALYTICAL BIOCHEMISTRY 217, 139–147 (1994)

Biotinylated and Cysteine-Modified Peptides as Useful Reagents for Studying the Inhibition of Cathepsin G

Abraham M. Brown,* Sam M. George,* Arthur J. Blume,† Russell G. Dushin,‡ J. Steven Jacobsen,* and June Sonnenberg-Reines*,[1]

*Departments of *CNS Biological Research, †Molecular Pharmacology, and ‡CNS Chemical Research, Medical Research Division, Lederle Laboratories, American Cyanamid Company, Pearl River, New York 10965*

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An assay for studying the proteolytic activity of endopeptidases using a biotinylated and cysteine-modified peptide has been developed. This assay is rapid, sensitive, and reproducible. Although used here specifically for the enzyme which cleaves at the amino terminus (N-terminus) of β-amyloid peptide (BAP); this type of radiolabeled substrate is readily applied to the analysis and detection of other endoprotease activities. This method relies on a peptide substrate which contains: (a) the amino acids flanking the enzymatic cleavage site, (b) an added cysteine at the carboxy-terminus to allow for incorporation of radiolabel via an addition reaction with tritiated *N*-[ethyl-1,2-^{3}H]maleimide (^{3}H-NEM), and (c) a biotin at the N-terminus to allow for binding to avidin-coated scintillation proximity assay (SPA) beads. It has been suggested that the enzyme involved in the N-terminal cleavage of amyloid precursor peptide to generate BAP is a chymotrypsin-like serine protease such as cathepsin G. To study this enzymatic activity and to screen for its inhibitors, we have synthesized the peptide biotin–SEVKMDAEFdC which contains the amino acids flanking the N-terminal cleavage site of BAP. Tritiated NEM is covalently bound to the cysteine at the carboxy-terminal end and the labeled peptide is purified by reverse-phase high-performance liquid chromatography. Following digestion of ^{3}H-NEM-labeled peptide by cathepsin G, the biotinylated side of the cleaved peptide is bound to the SPA bead, while the tritiated end of the cleaved peptide remains in solution. Enzymatic hydrolysis is measured as the loss of ^{3}H-induced scintillation signal. This method has allowed us to rapidly determine kinetic constants and develop a high throughput screen to study inhibition of cathepsin G cleavage in a native peptide context.

The β-amyloid protein (BAP)[2] is a proteolytic cleavage product of the amyloid precursor protein (APP) (1) that accumulates at high levels in the brains of Alzheimer's disease (AD) patients (2–4). Cleavage of APP within its BAP domain by the enzyme "secretase" (5,6) releases a soluble amino-terminal (N-terminal) APP fragment (7,8) which is identical to protease nexin 2 (9) and precludes the formation of BAP (10,11). Very little information is available about the identity of the endogenous enzymes involved in generating BAP. The use of peptide and modified reporter substrates for assaying protease activity has led to the proposal of various candidate enzymes as responsible for the N-terminal proteolytic cleavage of BAP (12–16) from APP.

A number of technical approaches have been employed to measure these and other proteolytic activities. One is the use of peptide substrates which contain a chromogenic or fluorogenic group attached to the carboxy-terminal (C-terminal) end of the peptide. Upon enzymatic cleavage this group is released into solution (13,15) and subsequently measured by spectrophotometric or fluorometric techniques. Another approach relies on the use of radiolabeled substrates which are separable from the resulting products by selective precipitation (17–19). Enzymatic activity is detected by measuring the amount of radiolabeled peptides released into

[1] To whom correspondence should be addressed. Fax: (914) 732-5537.

[2] Abbreviations used: BAP, β-amyloid protein; APP, amyloid precursor protein; AD, Alzheimer's disease; HPLC, high-performance liquid chromatography; SPA, scintillation proximity assay; ^{3}H-NEM, *N*-[*ethyl*-1,2-^{3}H]maleimide; TFA, trifluoroacetic acid; RT, room temperature; ACT, α-1-antichymotrypsin; PITC, phenylisothiocyanate.

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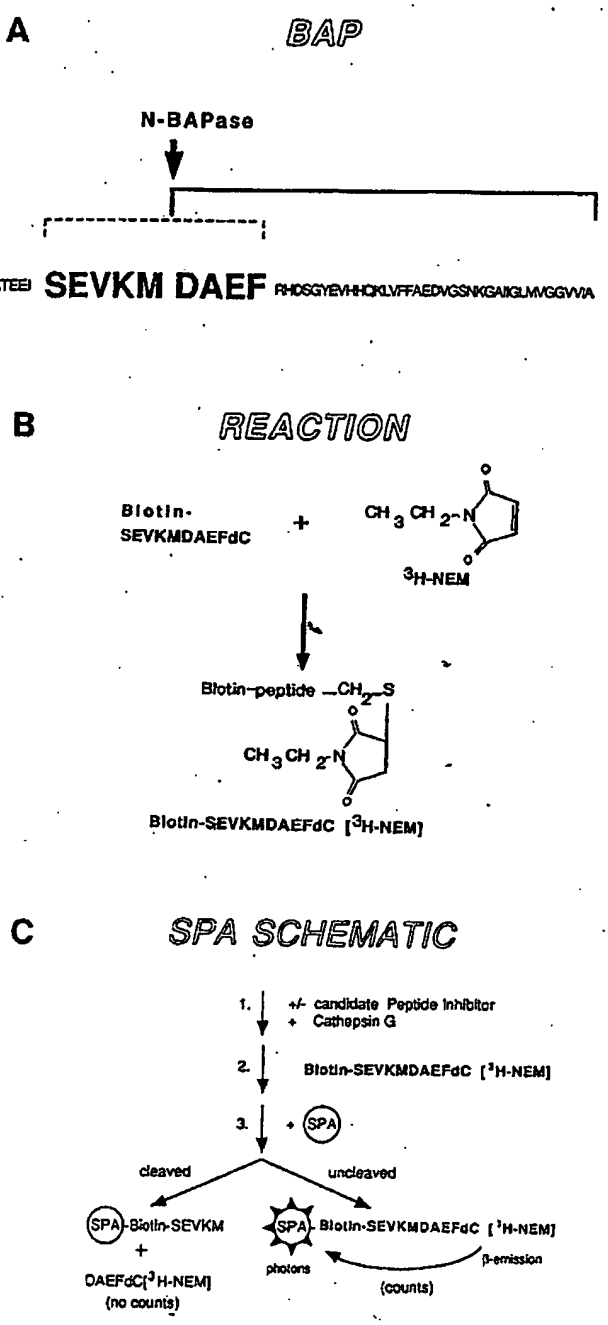

FIG. 1. Amino-terminal enzymatic activities associated with β-amyloid peptide (BAP). (A) The putative cleavage site for N-terminal BAP cleaving activity is between the methionine and aspartate (first amino acid of BAP) and is indicated by an arrow. The solid line indicates the 42-amino acid sequence of BAP. The dashed line indicates the amino acid sequence flanking the N-terminal cleavage site of BAP. (B) Tritiated or unlabeled *N*-ethylmaleimide reacts with the C-terminal cysteine amino acid. (C) Schematic illustration of the scintillation proximity assay. Upon binding of biotin-SEVKM-DAEFdC [^{3}H-NEM] to streptavidin, the fluorescer integrated into the SPA beads is placed in close enough proximity to the radiolabeled carboxy terminus to emit β-energy. Once the peptide is cleaved by cathepsin G, the energy emitted from the labeled carboxy terminus is dissipated to the buffer and the fluorescer is not activated.

the supernatant. Variants of this assay rely on the resolution of substrate from product either by thin-layer chromatography (20) or high-performance liquid chromatography (HPLC) (16,21). Enzymatic activity is measured as substrate loss or product formation.

An HPLC assay to characterize the enzymatic profile of various enzymes for the substrate SEVKMDAEF, a peptide which encompasses the N-terminal cleavage site of BAP, has been developed (16). However, the HPLC method is time consuming, labor intensive, and incompatible with high throughput screening of protease inhibitors. We have therefore developed an alternate approach to the HPLC method which utilizes modifications of two existing procedures. One approach places the cleavage site within a peptide which contains an N-terminal biotinyl group and a radioiodinated group (22) or a fluorescein group (23) at the carboxy terminus. Avidin permits the selective retrieval of cleaved and uncleaved biotinylated substrate and the enzymatic activity is measured as a function of released label into solution. Both iodination and fluorescein methods require the separation of product from substrate before the detection step.

Another approach, the scintillation proximity assay (SPA), does not require separation before detection and has been used extensively for receptor binding assays (24,25). In this paper we demonstrate the utility of SPA in a protease/inhibitor assay. The rationale for the assay is depicted in Fig. 1C. Biotinylated peptide is bound to SPA beads which are coated with streptavidin and contain a core of scintillant. The scintillant only emits photons when low-energy β-emitters such as ^{3}H are in close proximity (i.e., bound) to the bead. However, when a protease cleaves the substrate peptide, the radiolabeled end of the peptide is liberated from the biotin end and is dissipated into the solvent. Inhibition of this proteolysis will result in maintaining the radioactive moiety in close proximity to the scintillant and allowing the β-emission to be detected. Previous protocols called for the use of ^{125}I as the β source (22). However, the harsh conditions required for incorporation of iodine into tyrosine often result in oxidation of the biotin moiety.

We have therefore combined the positive attributes from each of these systems to develop a strategy which can be used for screening inhibitors of cathepsin G. This strategy uses a modified biotinylated and tritiated *N*-[*ethyl*-1,2-^{3}H]maleimide (^{3}H-NEM)-labeled peptide and relies on binding of the biotin-containing substrates and products by SPA beads. This assay can be performed in a single tube, since the separation of bound

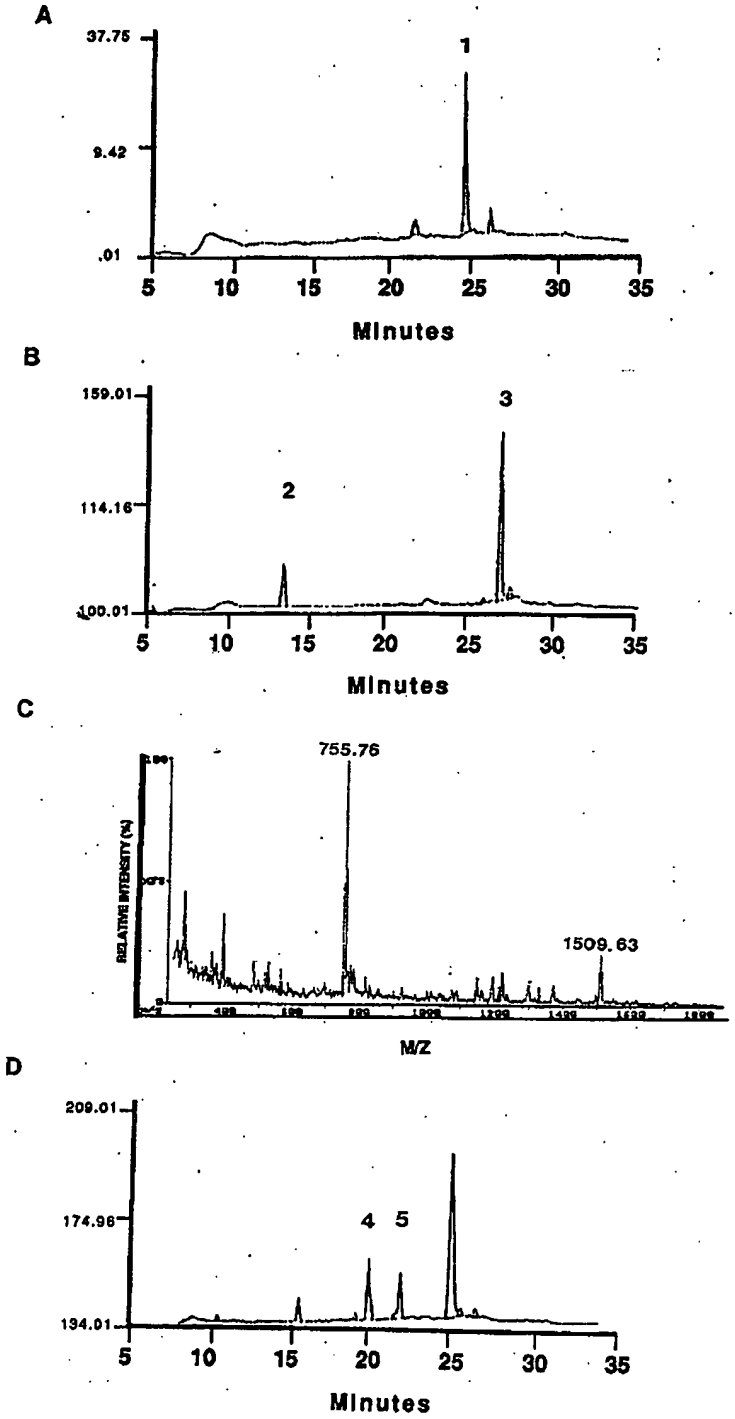

FIG. 2. HPLC separation of biotin–SEVKMDAEFdC from biotin–SEVKMDAEFdC [NEM]. (A) Biotin–SEVKMDAEFdC (peak 1) has a retention time of 24.5 min. (B) Forty micrograms of biotin–SEVKMDAEFdC (peak 1; retention time, 24.5 min) is reacted with 80 nmol of NEM (peak 2; retention time, 13.5 min). Biotin–SEVKMDAEFdC [NEM] (peak 3) has a retention time of 27 min. (C) Electrospray mass spectrometry of biotin–SEVKMDAEFdC [NEM] (peak 3). (D) Incubation of 5 μg biotin–SEVKMDAEFdC [NEM] (peak 3) with 0.4 μM cathepsin G for 15 min at 37°C results in the formation of biotin–SEVKM (peak 4) and DAEFdC [NEM] (peak 5).

and unbound tracer is not necessary. It also uses a low-energy radioactive label which is stable and eliminates the danger of high-energy γ- and β-emission from ^{125}I or ^{32}P, eliminates oxidization of amino acids within the substrate peptide, and is highly sensitive, accurate, reproducible, and compatible with the requirements for large-scale random screening of protease inhibitors.

MATERIALS AND METHODS

Peptide Labeling

Biotin–SEVKMDAEFdC (7.6 nmol; Penisula Labs, Belmont, CA) was labeled with 0.25 mCi of 44.4 Ci/mmol [^{3}H]NEM (New England Nuclear, Boston, MA) or 80 nmol NEM (Pierce, Rockford, IL). Nonradioactive NEM was reacted with biotin–SEVKMDAEFdC peptide in order to determine the retention time of biotin–SEVKMDAEFdC [NEM]. The procedure was as follows: 10 μg of biotin–SEVKMDAEFdC peptide in 40 μl of 200 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) buffer, pH 6.0, was added to 80 nmol NEM in 40 μl Mes, pH 6.0. After 1 h the unreacted peptide and NEM were separated from biotin–SEVKMDAEFdC [NEM] by reverse-phase HPLC on a C-18, 3.9 × 150-mm Delta Pak column (Waters, Milford, MA). HPLC was performed on a Waters 840 system equipped with a Model 712 WISP and two 510 HPLC pumps. A Lamda 490 spectrophotometer (Waters) was used to monitor uv absorbance at 214 nm. Separation of substrate from product was performed using a 50-min, 0–50% acetonitrile in 0.1% trifluoroacetic acid (TFA), linear gradient. The purified labeled peptide was stored at 4°C and is stable for at least 6 weeks.

Binding of Biotinylated Peptide to SPA Beads

Biotin–SEVKMDAEFdC [^{3}H-NEM], cleaved biotin–SEVKMDAEFdC [^{3}H-NEM], or biotin-[^{3}H] (Amersham, Arlington Heights, IL) in a total volume of 90 μl of 10 mM phosphate buffer, pH 6.0, was added to 10 μl of a solution containing 0.25 μl of SPA beads (Amersham) in phosphate-buffered saline (PBS), pH 7.4, and incubated for 20 min. β-Emission of bound peptide was determined by scintillation counting (Beckman A-LS 3801).

Enzymatic Cleavage of Biotin–SEVKMDAEFdC [^{3}H-NEM] by Cathepsin G

The 50-μl total reaction mixture contained 30 μl of 10 mM phosphate buffer, pH 6.0, and labeled peptide (4000 cpm or 2 ng peptide) diluted in 10 μl of 10 mM phosphate buffer, pH 6.0. Ten microliters of 68 nM (final concentration) cathepsin G (Athens Research, Athens, GA) was added to the peptide mixture and the reaction was incubated at room temperature (RT). The reaction was stopped by the addition of SPA beads in phosphate-buffered saline, pH 7.4. We have found that once the substrate is bound to the SPA beads it can no longer be cleaved by cathepsin G. β-Emission of bound peptide was determined by scintillation counting (Beckman A-LS 3801).

For determination of K_m and K_{cat} values, the rate of hydrolysis of biotin–SEVKMDAEFdC [^{3}H-NEM] (26 nM) and unlabeled SEVKMDAEF (1–113 μM) was measured by adding 68 nM cathepsin G (final concentration) to a final volume of 50 μl of 10 mM phosphate buffer, pH 6.0. The kinetic constants are then extracted from the Lineweaver–Burke plot of the data (26). All assays were performed at RT unless otherwise indicated.

HPLC Analysis of the Digestion of HSEVKMDAEF, SEVKMDAEF, and Biotin–SEVKMDAEFdC by Cathepsin G

HSEVKMDAEF and SEVKMDAEF were synthesized by the Department of Protein Chemistry at Lederle Laboratories (American Cyanamid Company, Pearl River, NY) and the purity and amino acid composition of the peptide was confirmed by electrospray ionization mass spectrometry as described (27). For determination of K_m and K_{cat} values, the rate of hydrolysis of HSEVKMDAEF was measured by adding 0.4 μM cathepsin G (final concentration) to 25–750 μM substrate in 10 mM Tris–HCl, pH 6.2, at 37°C. Substrates and products were then measured by reverse-phase HPLC for various incubation periods over a range of substrate concentrations. HPLC was performed using a 50-min, 0 to 50% acetonitrile in 0.1% TFA, linear gradient and identified either by comparing their retention times with authentic peptide standards and/or analyzing the collected peaks by amino acid sequencing. The kinetic constants were extracted as above (26).

For comparison of cleavage activity of SEVKMDAEF and biotin–SEVKMDAEFdC, 100 μM of each substrate was incubated for indicated times at 37°C with either 0.2 or 0.4 μM cathepsin G. The mixture of substrate and digestion products were analyzed by HPLC. Cleavage was measured as loss of substrate.

When α-1-antichymotrypsin (ACT) (Athens Research and Technology, Athens, GA) was used as an inhibitor, it was preincubated with enzyme in a 30-μl volume for 10 min at RT followed by addition of peptide.

Amino Acid Analysis and Mass Spectrometry of Cleavage Products

Product peaks were separated by reverse-phase HPLC, collected, and lyophilized. The amino acid analysis was performed as previously described (28) using a Pico Tag work station (Water Associates, Milford, MA) which employs phenylisothiocyanate (PITC) derivatiza-

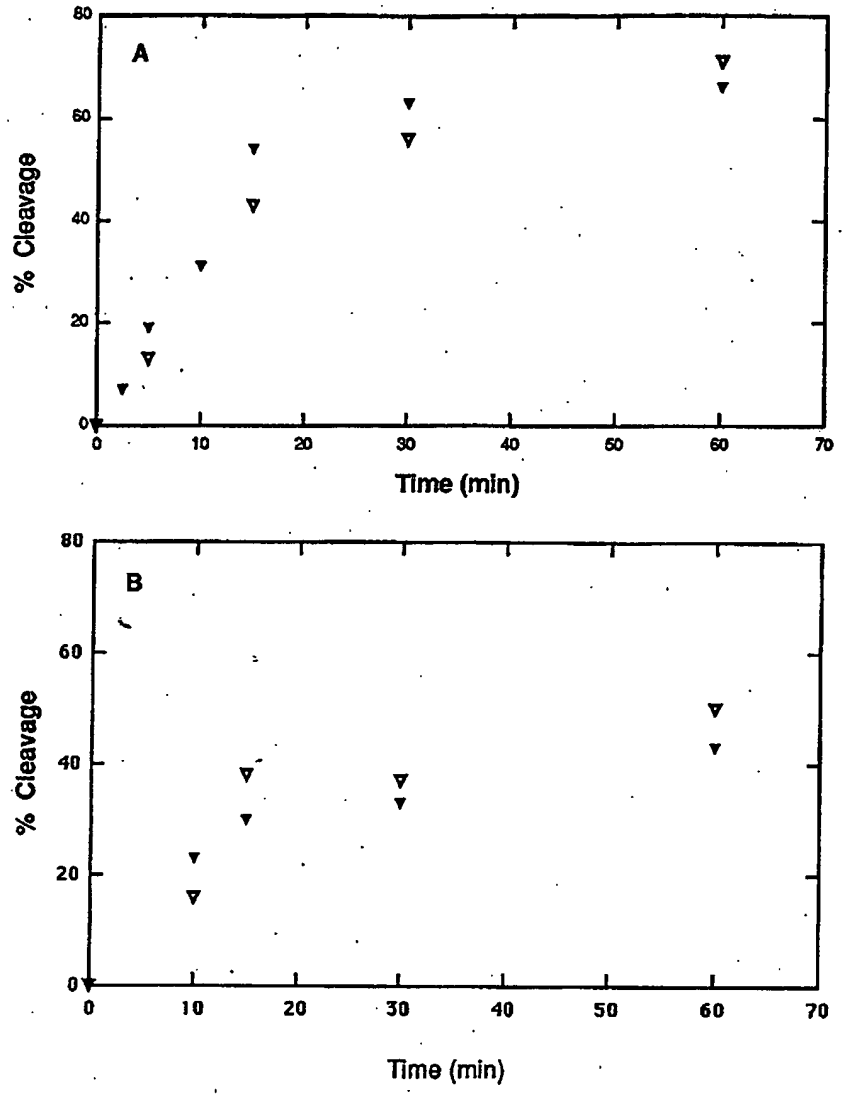

FIG. 3. Comparison of cleavage activity of biotin–SEVKMDAEFdC and SEVKMDAEF for the enzyme cathepsin G. The rate of hydrolysis of (▽) biotin–SEVKMDAEFdC and (▼) SEVKMDAEF was measured by adding a final concentration of (A) 0.4 or (B) 0.2 μM cathepsin G to 100 μM substrate in 10 mM phosphate, pH 6.0, at 37°C. Reverse-phase HPLC was performed using a linear gradient of 0 to 50% acetonitrile in 0.1% TFA for 50 min.

tion of amino acids. Peaks fractions for electrospray ionization mass spectrometry were analyzed on a SCIEX (Thornhill, Ontario, CAN), API III triple quadrupole mass spectrometer equipped with a pneumatically assisted electrospray ionization source as described (27).

RESULTS

Labeling Biotin–SEVKMDAEFdC with N-Ethylmaleimide

Biotin–SEVKMDAEFdC used in these studies contains the putative cleavage site between the methionine (Met) and the first aspartate (Asp) residue of BAP (Fig. 1A). The cysteine addition allows for radiolabeling of the substrate with ^{3}H-NEM via the sulfhydryl group (Fig. 1B) while the D-isomer of cysteine is used to retard exopeptidase degradation at the c-terminus of the peptide (Figs. 1B and 1C). The separation of biotin–SEVKMDAEFdC [NEM] peptide from free NEM is depicted in Fig. 2. HPLC analysis reveals that NEM has a retention time of 13.5 min (Fig. 2B, peak 2); unlabeled peptide, 24.5 min (Fig. 2A, peak 1); and the NEM adduct, 27 min (Fig. 2B, peak 3). The peak at 27 min (Fig. 2B, peak 3) was collected and analyzed by electrospray

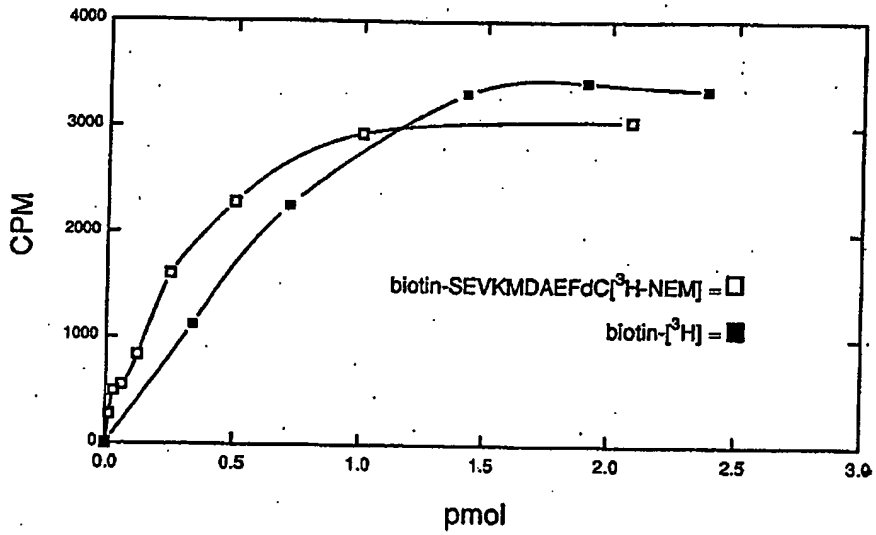

FIG. 4. Binding of biotin-[^{3}H] and biotin–SEVKMDAEFdC [^{3}H-NEM] to scintillation proximity beads. Varying amounts of biotin-[^{3}H] and biotin–SEVKMDAEFdC [^{3}H-NEM] in a total volume of 90 μl of 10 mM phosphate buffer, pH 6.0, is added to 10 μl of 0.25 μl SPA beads in phosphate-buffered saline, pH 7.4, and incubated for 20 min.

ionization mass spectrometry (Fig. 2C) to confirm the presence of biotin–SEVKMDAEFdC [NEM] (M + 1 of 1509.63) and absence of unreacted peptide, biotin–SEVKMDAEFdC (M + 1 of 1383.89). The 27-min retention time was then used as standard to isolate the radiolabeled biotin–SEVKMDAEFdC [^{3}H-NEM].

Cleavage of Labeled Peptide by Cathepsin G

We have previously shown that when HSEVKMDAEF is digested at pH 6.2 with cathepsin G, 70% of the products formed are the result of Met/Asp (M/D) cleavage (16). Biotin–SEVKMDAEFdC [NEM] is also susceptible to M/D cleavage by cathepsin G. Peptides isolated by the HPLC as peaks 4 and 5 (Fig. 2D) were analyzed by PITC amino acid analysis. These results indicate that peak 4 is SEVKM and peak 5 is DAEFdC, confirming a cleavage between M/D of the substrate. There is also some cleavage between K/M at basic pH, but the predominant cleavage (>50%) is M/D at pH 6.2 and below. Thus the addition of cysteine and biotin to the ends of the peptide does not significantly alter its pH-dependent cleavage specificity of cathepsin G (16).

The similarity of biotin- and cysteine-containing substrates to peptides containing native sequences is more clearly shown in Fig. 3. Biotin–SEVKMDAEFdC and SEVKMDAEF (100 μM) were digested with 0.2 and 0.4 μM of cathepsin G for the times indicated and product formation was analyzed by HPLC. Both substrates display similar digestion kinetics at both input concentrations of enzyme.

Specific Binding of Biotinylated Peptide to Scintillation Proximity Assay Beads

The biotin modification at the amino terminus provides a ligand for binding of the peptide to a streptavidin-coated SPA bead. Biotin–SEVKMDAEFdC [^{3}H-NEM] binds to the SPA beads with a maximum binding of 1.0 pmol peptide/0.25 μl beads (Fig. 4). Binding is very similar to that of biotin-[^{3}H] which is 1.42 pmol/0.25 μl beads, indicating that biotinylated peptide has the same access to the resin as free biotin. The reaction is specific since biotin can displace 100% of the biotin–SEVKMDAEFdC [^{3}H-NEM] from the SPA beads (data not shown).

The rate of hydrolysis of biotin–SEVKMDAEFdC [^{3}H-NEM] (26 nM) and unlabeled SEVKMDAEF (10 μM) was measured by adding 68 nM cathepsin G (final concentration) to a final volume of 50 μl 10 mM phosphate buffer, pH 6.0, and incubated for 10 min at RT. Using these conditions the assay is reproducible (intraassay variability ± 5.0%, interassay variability ± 8.4%), and is linear with time (Fig. 5A), enzyme concentration (Fig. 5B), and substrate concentration (Fig. 6). As cathepsin G is increased, the maximum enzyme concentration to observe linearity of cleavage is 200 nM (Fig. 5B). The assay is also linear with increasing substrate concentrations and has a K_m of 72 μM and a K_{cat} of 0.36 (Table 1, Fig. 6). Kinetic analysis was performed using a Lineweaver–Burke plot of the reciprocal of the rate of product formed against the reciprocal of the initial substrate. As expected, when comparing the HPLC and SPA assays, the K_m and K_{cat} for both assays are similar (Table 1).

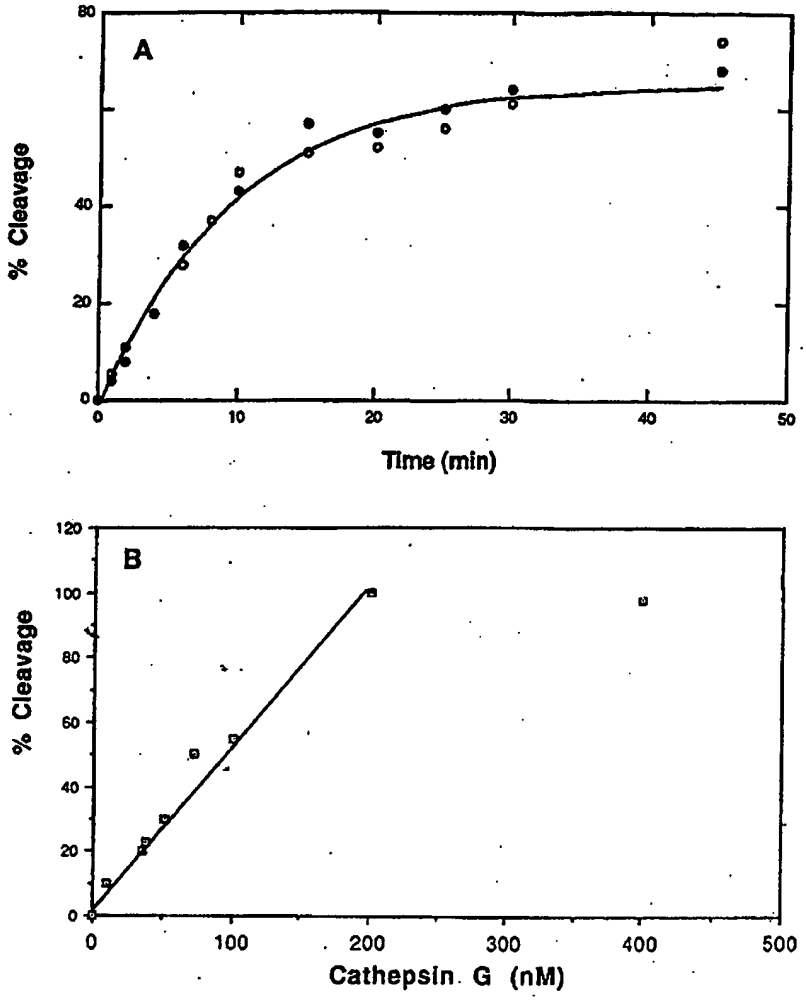

FIG. 5. Enzymatic cleavage of biotin–SEVKMDAEFdC [^{3}H-NEM] by cathepsin G. (A) Cathepsin G (68 nM) was incubated with biotin–SEVKMDAEFdC [^{3}H-NEM] (26 nM) plus SEVKMDAEF (10 μM) at various time points. (○) Hollow and (●) filled circles represent separate experiments. The line is a best fit to an exponential rise. (B) Cathepsin G (0 to 400 nM) was added to biotin–SEVKMDAEFdC [^{3}H-NEM] (26 nM) plus SEVKMDAEF (10 μM) in 50 μl of 10 mM phosphate buffer, pH 6.0, and incubated for 10 min at RT. The line is a linear regression fit to the first eight points.

ACT, a specific serine protease inhibitor and an integral component of neuritic plaques in Down's syndrome and AD (29), is a potent inhibitor of cathepsin G in the HPLC assay (16). We now demonstrate that ACT also inhibits cathepsin G as measured by the SPA assay. Enzyme was preincubated for 10 min with ACT prior to addition of biotin–SEVKMDAEFdC [^{3}H-NEM] and an K_i value of 61 nM (Table 1) was obtained. The K_i of the HPLC assay (82 nM) is slightly higher when compared to that of the SPA assay (61 nM).

DISCUSSION

We have developed a general radiometric assay for studying the proteolytic activities of endopeptidases using a tritiated–biotinylated peptide. The method relies on the use of a peptidyl substrate incorporating susceptible bonds located between a biotinyl group at one end and a radiolabeled NEM group at the other. The presence of the biotin moiety on the N-terminal end of the substrate allows the intact peptide and/or N-terminal portion of the cleaved peptide product to be retrieved by streptavidin-coated scintillation proximity assay beads. If the ^{3}H label remains on the captured peptide, the weak β particles emitted by the ^{3}H will excite the scintillant contained within the bead. Enzymatic activity is measured by the release into solution of the radiolabeled C-terminal peptide fragment which reduces photon emission and scintillation counts. SPA beads are preferable to streptavidin plates since the former

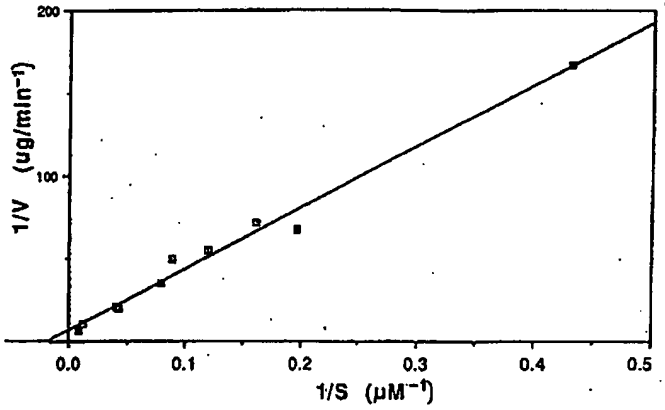

FIG. 6. Lineweaver–Burk plot of reciprocal rate against reciprocal substrate concentration. For determination of K_m and K_{cat} values, the rate of hydrolysis of biotin–SEVKMDAEFdC [^{3}H-NEM] (26 nM) and unlabeled SEVKMDAEF (1–113 μM) was measured by adding 68 nM cathepsin G (final concentration) to a final volume of 50 μl 10 mM phosphate buffer, pH 6.0, at RT. The line is a linear regression fit to the data.

method does not require washing to remove ^{3}H from solution and eliminates the need for scintillation fluid.

The present study demonstrates the usefulness of radiolabeling an amino terminal biotinyl peptide via addition of cysteine at its c-terminus. Free C-terminal thiol can be labeled with ^{3}H-NEM, fluorescein, or rhodamine using nonoxidizing conditions, and then the peptide can be detected by scintillation, fluorescence, coloriometric, or antibody techniques. The feasibility of the fluorescein approach has actually been tested for the identification of HIV-1 protease inhibitors (23). Fluorescein was covalently linked to a lysine rather than a cysteine at the C-terminal end of the molecule. The use of linkage through lysine was not compatible with our substrate since there is a lysine within the cleavage site of the peptide which is critical for enzyme/substrate binding.

Another approach by Basak *et al.* (22) also involves a peptide substrate which contains a biotin moiety on one side of the cleavage site, but tyrosine instead of a cysteine is added at the C-terminal end to allow for the radioiodination of the substrate. Our initial attempts to radiolabel our peptide through a C-terminal tyrosine using any of the common iodination procedures, namely chloromine-T, lactoperoxidase, or iodo-beads (Pierce) led to two major problems. First, vigorous iodination of the biotinylated peptide results in the complete loss of the avidin binding property (data not shown), similar to Green (30), who also found a much decreased affinity of the biotin function toward avidin. This may be due to the oxidation of the biotinyl sulfur atom to its sulfoxide and sulfone forms (31). Second, the iodination procedure results in oxidation of Met and we find that cathepsin G is unable to cleave oxidized SEVKMDAEF (data not shown).

Selection of the target sequence, the size of the peptide, and the spacer arm linking the biotin function to the cleavage site are among the many parameters which can influence cleavage specificity. Sequence analysis of both cerebrospinal fluid and conditioned media of cells expressing APP (32–34), as well as cerebrovascular BAP from AD or Down's syndrome brain (2) all indicate that the cleavage responsible for the formation of the free N-terminus of BAP from APP occurs most likely between the Met and Asp residue (codon 671/672 of the APP751 transcript). Therefore we have used the residues flanking this cleavage site as our substrate peptide. The 10-amino acid peptide, biotin–SEVKMDAEFdC was selected on the basis of the following considerations. First the peptide flanks the BAP region and we know from other studies that it is cleaved between M/D by chymotrypsin-like serine proteases (12,16). Second, the minimum peptide requirement for 50% cleavage activity at the M/D site is the 6 mer VKMDAE (R. G. Dushin and J. Sonnenberg-Reines, unpublished data). We chose to elongate the peptide to SEVKMDAEF so that the biotin and the cysteine moiety are spaced far apart from the cleavage site, thereby limiting restrictions of enzyme/substrate binding. Third, addition of the biotin on the N-terminus during solid-phase synthe-

TABLE 1

Comparison of Kinetic Properties of Biotin–SEVKMDAEFdC [^{3}H-NEM] and HSEVKMDAEF Digested with Cathepsin G using the SPA and HPLC Assays

Assay	Enzyme (nM)	K_m (μM)	K_{cat} (s^{-1})	K_i (nM)
HPLC	400	85 ± 12[a]	0.21 ± 0.01[a]	82 ± 2[c]
SPA	68	72 ± 6[b]	0.36 ± 0.04[b]	61 ± 11[d]

Note. Values are means ± SEM.

[a] For determination of K_m and K_{cat} values, the rate of hydrolysis of HSEVKMDAEF was measured by adding a final concentration of 0.4 μM cathepsin G to 25–750 μM substrate in 10 mM Tris, pH 6.2, at 37°C. Reverse-phase HPLC was performed using a linear gradient of 0 to 50% acetonitrile in 0.1% TFA in 50 min.

[b] For determination of K_m and K_{cat} values, the rate of hydrolysis of biotin–SEVKMDAEFdC [^{3}H-NEM] (26 nM) and unlabeled SEVKMDAEF (1–113 μM) was measured by adding a final concentration of 0.068 μM cathepsin G to a final volume of 50 μl of 10 mM phosphate buffer, pH 6.0, at RT.

[c] Enzyme was preincubated with inhibitor for 10 min at RT prior to the addition of HSEVKMDAEF (100 μM). Samples were incubated at 37°C for 15 min in 10 mM Tris, pH 6.2.

[d] Enzyme was preincubated with inhibitor for 10 min at RT prior to the addition of biotin–SEVKMDAEFdC [^{3}H-NEM] (26 nM) and unlabeled SEVKMDAEF (10 μM). Samples were incubated at RT for 10 min in 10 mM phosphate, pH 6.0.

sis eliminates the need to biotinylate the peptide following labeling. Moreover, biotinylation via nucleophilic displacement may result in modification of the internal lysine which is close to the active site and may be needed for peptide/enzyme binding.

Since this assay is used as a high throughput screen, cost effectiveness is of major concern. As SPA beads are the costliest reagent in the assay, their input has been reduced to 0.5 μl/sample, allowing for 10,000 assays per kit, and radiolabeled peptide has been kept at a minimum (26 nM). Since the radiolabeled peptide (26 nM) is well below the K_m (Table 1, Fig. 6) of the reaction, additional SEVKMDAEF substrate was added to the reaction. SEVKMDAEF is a nonbiotinylated substrate which is digested with kinetics comparable to those of the radiolabeled peptide (Fig. 3). The ratio of substrate to enzyme is now increased by 75-fold and has the advantage of decreasing the cost of the labeled peptide and the amount of SPA beads necessary to bind the biotin peptide.

The results of the assay are quite comparable to those of the HPLC previously described (16). We have used the SPA assay initially to verify a known inhibitor of cathepsin G (i.e., ACT) and will now be screening for other inhibitors of this enzymatic activity. Although specifically used here for an enzyme which cleaves at the N-terminus of BAP, this type of labeled peptide substrate is readily applied to the detection of other endoprotease activities, as well as inhibitors of these activities.

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An ESP1/PDS1 Complex Regulates Loss of Sister Chromatid Cohesion at the Metaphase to Anaphase Transition in Yeast

Rafal Ciosk,* Wolfgang Zachariae,*
Christine Michaelis,* Andrej Shevchenko,†
Matthias Mann,† and Kim Nasmyth*‡
*Research Institute of Molecular Pathology
Dr. Bohr-Gasse 7
A-1030 Vienna
Austria
†European Molecular Biology Laboratory
Meyerhofstrasse 1
D-69012 Heidelberg
Germany

Summary

Cohesion between sister chromatids during G2 and M phases depends on the "cohesin" protein Scc1p (Mcd1p). Loss of cohesion at the metaphase to anaphase transition is accompanied by Scc1p's dissociation from chromatids, which depends on proteolysis of Pds1p mediated by a ubiquitin protein ligase called the anaphase promoting complex (APC). We show that destruction of Pds1p is the APC's sole role in triggering Scc1p's dissociation from chromatids and that Pds1p forms a stable complex with a 180 kDa protein called Esp1p, which is essential for the dissociation of Scc1p from sister chromatids and for their separation. We propose that the APC promotes sister separation not by destroying cohesins but instead by liberating the "sister-separating" Esp1 protein from its inhibitor Pds1p.

Introduction

Chromosome duplication during S phase produces sister chromatids that are held together by specific chromosomal proteins called cohesins (Guacci et al., 1997; Michaelis et al., 1997). Sister chromatids are later pulled to opposite poles of the cell during anaphase by microtubules that connect sister kinetochores to opposite poles of the mitotic spindle. The "splitting" force exerted by these microtubules is initially counteracted by cohesion between sisters, and the balance of these two forces results in chromosome alignment in the middle of the cell during metaphase (Nicklas, 1988). Loss of cohesion rather than an increase in the splitting force exerted by microtubules is thought to trigger disjunction of sisters at the metaphase to anaphase transition (Miyazaki and Orr-Weaver, 1994).

The properties of a cohesin called Scc1p or Mcd1p in *Saccharomyces cerevisiae* suggest how cohesion might be lost. Scc1p binds to chromosomes during S phase, it prevents premature separation of sister chromatids during G2/M, and disappears from chromosomes at the metaphase to anaphase transition (Michaelis et al., 1997). Thus, Scc1p's disappearance from chromosomes could be responsible for the separation of sister chromatids during anaphase. A key question is what causes the sudden disappearance of Scc1p and the loss of sister chromatid cohesion.

Cyclin proteolysis coincides with the metaphase to anaphase transition and depends on a large protein complex called the anaphase promoting complex (APC), which mediates ubiquitination of cyclins via destruction boxes close to their N termini (King et al., 1995; Sudakin et al., 1995; Zachariae and Nasmyth, 1996a). Inactivation of any one of at least ten APC subunits prevents not only cyclin destruction but also sister chromatid separation (Irniger et al., 1995; Zachariae et al., 1996b, 1998). Because cyclin destruction per se is not required for sister separation (Holloway et al., 1993; Surana et al., 1993), it has been proposed that the APC also mediates the destruction of proteins that act as inhibitors of anaphase. What might these proteins be?

One possibility is that degradation of cohesins facilitates loss of sister chromatid cohesion. Scc1p is an APC substrate that is destroyed during anaphase. Furthermore, loss of Scc1 function allows sister chromatids to separate in the absence of the APC activity. However, proteolysis of Scc1p commences at the onset of anaphase and is not completed until after chromosomes have reached opposite poles of the cell, which might be too late to trigger separation (Michaelis et al., 1997).

Another candidate is a protein called Pds1p, which is required for preventing anaphase when DNA or spindles are damaged (Yamamoto et al., 1996b). Pds1p is normally destroyed by the APC around the metaphase to anaphase transition (Cohen-Fix et al., 1996). Furthermore, mutant variants of Pds1p, which cannot be degraded due to mutations in their destruction boxes, block the separation of sister chromatids. This strongly suggests that destruction of Pds1p is necessary for the separation of sister chromatids.

Results described here and by Yamamoto et al. (1996b) imply that the APC promotes sister separation and dissociaton of Scc1p solely through destruction of Pds1p. We purified Pds1p and found it tightly associated with a 180 kDa protein, which was identified by mass spectrometric sequencing as the product of the *ESP1* gene (McGrew et al., 1992). We show that Esp1p is essential for the separation of sister chromatids and for the dissociation of Scc1p from all regions of chromosomes. Our data imply that the APC mediates sister chromatid separation not by degrading cohesins but by liberating the "sister-separating" Esp1 protein from an inhibitory embrace by its guardian Pds1p. Esp1p is related to fission yeast Cut1p, which is required for chromosome segregation, and associates with a protein destroyed by the APC called Cut2p (Uzawa et al., 1990; Funabiki et al., 1993, 1996a, 1996b). These parallels suggest that sister separation may be triggered by a similar mechanism in all eukaryotic cells.

Results

The APC's Sole Role in Separating Sisters Is to Destroy Pds1p

Temperature-sensitive (ts) *apc* mutant cells arrest uniformly in metaphase when shifted to 37°C. However,

‡To whom correspondence should be addressed.

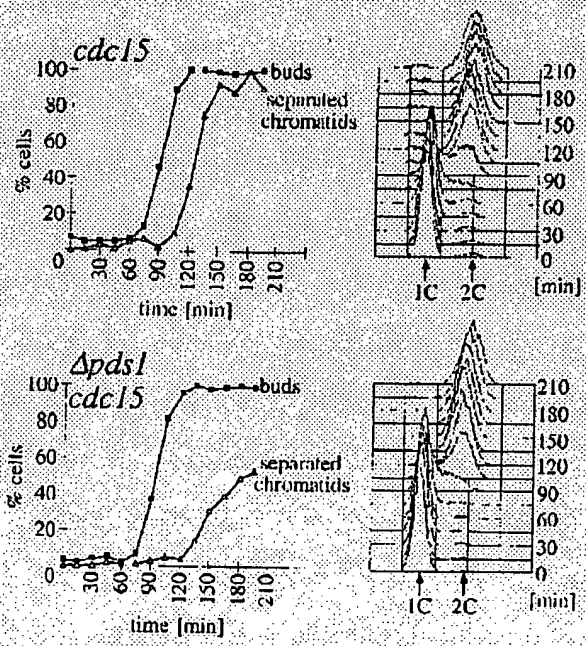


Figure 1. Pds1p Is Required for Efficient Separation of Sister Chromatids

Small G1 cells of *cdc15-2* (K7173) and *cdc15-2 pds1Δ* (K7191) strains, both having cenV marked by GFP (cenV-GFP), were inoculated into medium at 37°C. Left, the fraction of budded cells and cells with two separated GFP "dots" (i.e., separated sister chromatids at cenV). Right, cellular DNA content measured by flow cytometry (FACS). Comparing the kinetics of sister separation in different mutant strains is complicated by differences in their ability to undergo cytokinesis and rereplicate their genomes. It was to alleviate this problem that we employed a *cdc15* mutation (Culotti and Hartwell, 1971), which does not affect the kinetics of sister separation (data not shown) but prevents cytokinesis and DNA rereplication. It was not needed for analyzing *apc* mutants, which fail anyway to cytokinese and rereplicate.

deletion of *PDS1* allows between 33%–67% of ts *apc* mutant cells to undergo nuclear division at the restrictive temperature 3 hr after removal from hydroxyurea (HU) caused arrest (Yamamoto et al., 1996b). These data raise the possibility that destruction of Pds1p might be the APC's sole function in promoting sister chromatid separation. To test this, we compared the kinetics of sister chromatid separation in wild-type, *pds1Δ*, *apc*, and *pds1Δ apc* double mutants as unbudded G1 cells isolated by centrifugal elutriation were incubated at 37°C. We followed sister separation in these cultures by visualizing tetracycline repressor-GFP fusion proteins, bound to multiple tet operators integrated 30 kb from the centromere of chromosome V (Michaelis et al., 1997).

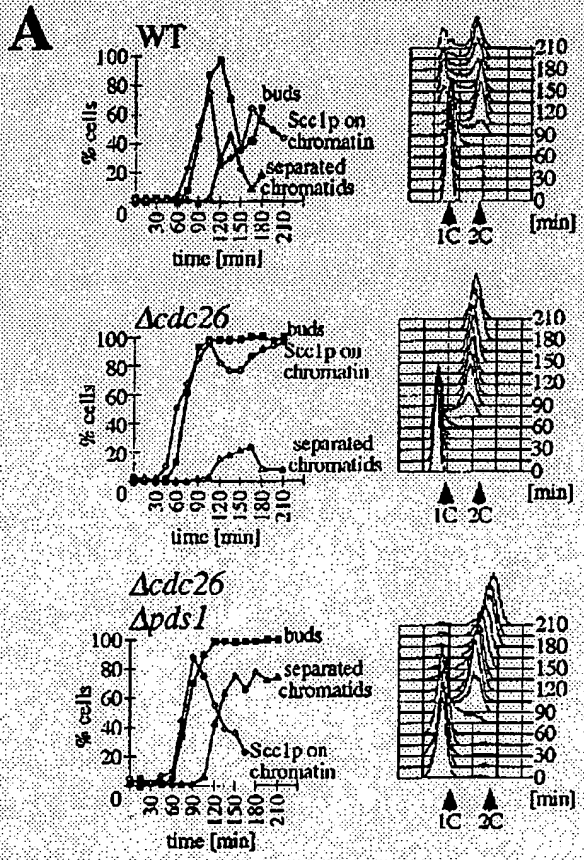
In *PDS1 cdc15-2* cells (i.e., wild type; see legend to Figure 1), sister separation occurs 30–35 min after cells have produced a bud. In *pds1Δ cdc15-2* cells, however, sister separation does not commence until 60 min after budding and is delayed for even longer in many cells (Figure 1). Mutations in genes encoding APC subunits (such as *CDC26* and *APC2*) have a much more severe effect. Little or no sister separation occurs in *apc2-1* or *cdc26Δ* mutants at 37°C (Zachariae et al., 1998). Remarkably, the kinetics of sister separation are very similar, if not identical, in *pds1Δ cdc15-2* and in *pds1Δ apc2-1* mutants (Figure 1; Zachariae et al., 1998). The implication is that deletion of *PDS1* causes the APC to become redundant for sister separation. Similar data were obtained with *pds1Δ cdc26Δ* double mutants (data not shown). Although *pds1Δ apc* double mutant cells separate sister chromatids, they fail to destroy the mitotic cyclin Clb2p, to disassemble their mitotic spindle, to undergo cytokinesis, and to rereplicate their genome (Yamamoto et al., 1996b; Zachariae et al., 1998).

Scc1p Dissociates from Chromosomes in *apc* Mutants Lacking Pds1p

To test whether APC-dependent destruction of Pds1p is also sufficient to trigger Scc1p's dissociation from chromosomes, we measured the association of Scc1myc18p with chromatin in "chromosome spreads" prepared from wild-type, *cdc26Δ*, and *pds1Δ cdc26Δ* double mutant cells as they progressed through the cell cycle at 37°C. Whereas Scc1p did not dissociate from chromatin in *cdc26Δ* single mutants, it dissociated from chromatin in *pds1Δ cdc26Δ* double mutants with kinetics that were similar to wild type (Figure 2A). It is possible to visualize simultaneously the GFP signal marking chromosome V (30 kb away from its centromere) and the presence of Scc1myc18p marked by Cy3. In both wild-type and *pds1Δ cdc26Δ* double mutant cells, Scc1p vanishes from chromosomes by the time that sister centromeres disjoin, whereas it never dissociates in *cdc26Δ* mutants that fail to separate sisters (Figure 2B). These data suggest that the APC's sole task in promoting dissociation of Scc1p from chromosomes during anaphase is to mediate destruction of Pds1p.

Pds1p Forms a Stable Complex with a 180 kDa Protein Encoded by the *ESP1* Gene

We failed to detect an association between the bulk of Pds1 protein and chromatin using the same chromosome spreading technique used to demonstrate Scc1p's association. The vast majority of Pds1p is washed away to an extent similar to nonchromosomal nuclear proteins like the exportin Cse1p (Tanaka et al., 1997). To investigate how Pds1p might block sister chromatid separation without binding to chromatin, we analyzed whether Pds1p associates with other proteins. Protein extracts were prepared from *PDS1myc18* cells grown in the presence of ^{35}S methionine and subjected to immunoprecipitation with an antibody to the myc-epitope (Figure 3A). A protein of 180 kDa was detected in immunoprecipitates from *PDS1myc18* strains but not from strains expressing non-tagged Pds1p or other myc-tagged proteins such as Cse1p or the APC subunit Apc2p. The Pds1p-p180 complex was purified by a large-scale immunoprecipitation from unfractionated whole-cell extracts, which yielded sufficient material to detect p180 on gels stained with silver. After in-gel digestion with trypsin, the recovered peptide mixture was analyzed by nano-electrospray tandem mass spectrometry (Mann and Wilm, 1995; Wilm and Mann, 1996). Sequences derived from four different peptides unambiguously identified p180 as the product of the *ESP1* gene (Figures 3B and 3C). We estimate that the molar ratio of Pds1myc18p to Esp1p



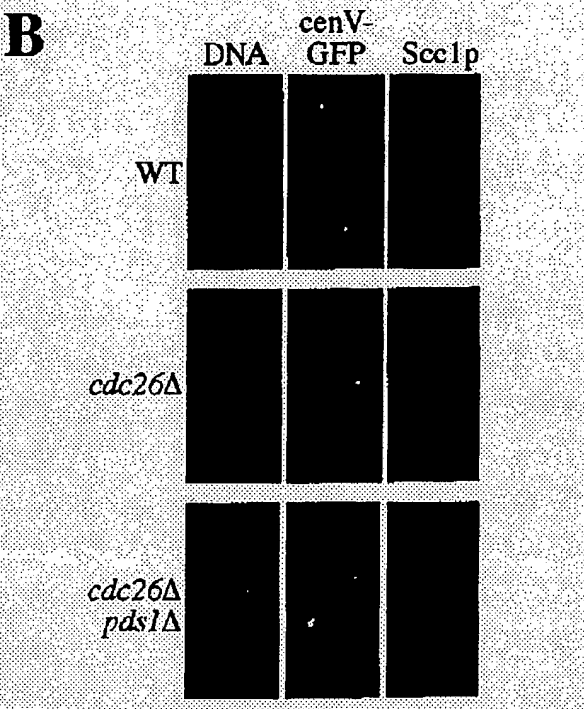


Figure 2. Deletion of the *PDS1* Gene Allows Sister Chromatid Separation and Dissociation of Scc1p from the Chromatin in the Absence of APC Function

(A) Dissociation of Scc1p from the chromatin in *cdc26Δ pds1Δ* cells. Small G1 cells of wild-type (K7056), *cdc26Δ* (K7042), and *cdc26Δ pds1Δ* (K7026) strains containing *SCC1myc18* and cenV-GFP were incubated at 37°C. Scc1myc18p associated with chromatin was detected by indirect immunofluorescence on chromosome spreads.

(B) Chromosome spreads of cells taken at 135 min. DNA stained with DAPI. The centromeric region of chromosome V visualized by GFP (cenV-GFP). Scc1myc18p associated with chromatin detected by indirect immunofluorescence after chromosome spreading. Note that the delay in sister separation caused by loss of Pds1p is partly alleviated by the myc tag on Scc1p, which possibly reduces Scc1p's ability to maintain sister chromatid cohesion.

was 1 to 0.4 in immunoprecipitates prepared from ^{35}S-labeled cells, which suggests that a sizeable fraction of soluble Pds1p is bound to Esp1p. We confirmed the association between Esp1p and Pds1p using a strain expressing Esp1myc18p and Pds1HA6p. Pds1HA6p was detected by Western blotting in anti-myc immunoprecipitates prepared using myc-specific antibody, both from wild-type and *cdc26Δ* mutant cells arrested at 37°C (Figure 3D).

Esp1p Is Required for Sister Chromatid Separation

esp1 mutants were first identified because they accumulate extra spindle pole bodies (Baum et al., 1988). When G1 cells of an *esp1-1* strain are incubated at the restrictive temperature, they duplicate DNA and form mitotic spindles; they then fail to segregate chromosomes but nevertheless proceed with cyclin destruction, cytokinesis, and genome reduplication (McGrew et al., 1992; Surana et al., 1993). To investigate whether Esp1p is needed for sister separation, we compared the kinetics of sister separation in *esp1-1 cdc15-2* double mutants with *cdc15-2* single mutants (i.e., "wild type"), as unbudded G1 cells isolated by elutriation were incubated at 37°C. This showed that *esp1-1* mutant cells separate centromere proximal sequences very inefficiently (Figures 1 and 4A). *esp1-1* mutant cells were equally defective in separating sequences 30 kb from the right hand telomere of chromosome V (data not shown). *esp1-1* mutants' failure to separate sisters is clearly more severe than that of *pds1Δ* mutants. This phenotype was confirmed using time-lapse video microscopy of live cells, which showed that paired sister chromatids in *esp1-1* mutants were pulled from one to the other side of the nucleus at the time when disjunction should have occured (data not shown). The lack of sister separation in *esp1-1* mutant cells is not due to activation of the Mad/Bub mitotic spindle surveillance mechanism (checkpoint) because neither *mad2Δ esp1-1* nor *bub2Δ esp1-1* double mutants separated sisters any more efficiently than *esp1-1* single mutants (data not shown).

Pds1p Destruction Occurs Normally in *esp1* Mutants

One explanation for the lack of sister separation in *esp1-1* mutants is that they fail to destroy Pds1p. We therefore used indirect immunofluorescence to compare the kinetics of disappearance of a myc-tagged Pds1 protein as wild-type and *esp1-1* G1 cells isolated by centrifugal elutriation are incubated at 37°C. Pds1p disappeared with similar kinetics in wild-type and *esp1-1* mutant cells (Figure 4B). The mutant cells transiently accumulate with unseparated sister chromatids

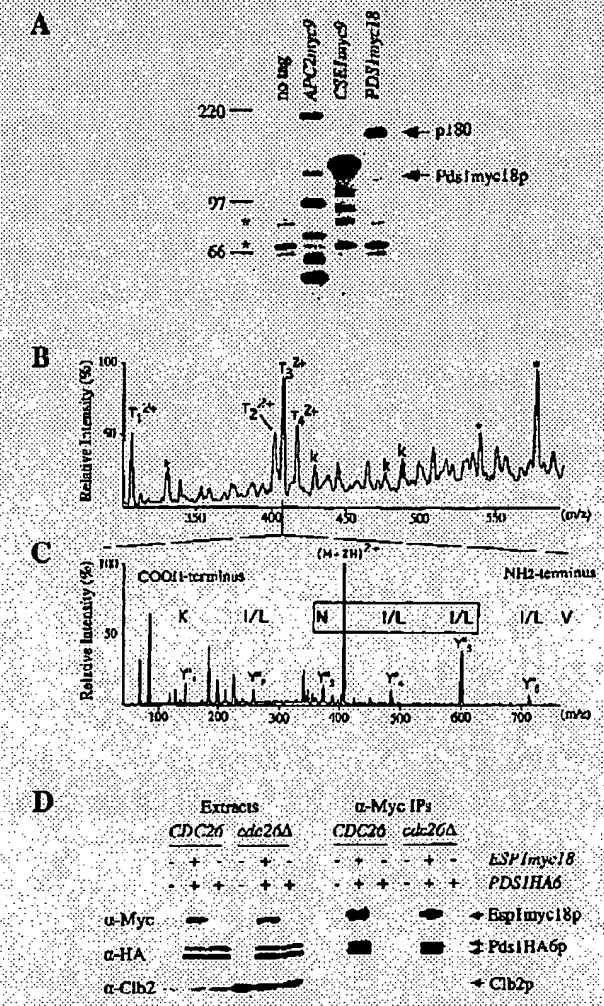


Figure 3. Pds1p Forms a Complex with Esp1p

(A) Coimmunoprecipitation of a 180 kDa protein with Pds1p. Control cells (no tag) and cells containing myc-tagged versions of *APC2*, *CSE1*, and *PDS1* were grown in medium containing ^{35}S methionine and cysteine. Protein extracts were immunoprecipitated with an antibody specific for the myc epitope. Bound proteins were separated on SDS-polyacrylamide gels and detected by fluorography. Proteins whose precipitation does not depend on the myc epitope are marked by asterisks. To identify p180 by nano-electrospay tandem mass spectrometric sequencing, immunoprecipitates prepared from unlabeled *PDS1myc18 Δpep4* cells were separated on a SDS-polyacrylamide gel followed by silver staining.

(B) Part of the mass spectrum of the peptide mixture extracted after in-gel digestion of the 180 kDa band with trypsin. The spectrum was acquired in parent ion scan mode, which detects peptide ions present in very low amounts by scanning for a fragment ion specific for peptides but not for the background (Wilm et al., 1996a). Ions are detected that yield, upon collisional fragmentation, daughter ions with a mass to charge ratio (m/z) of 86, the immonium ions of leucine and isoleucine. Tandem mass spectra were aquired upon fragmentation of selected peaks. Peaks were identified as trypsin autolysis products (*), peptides from human keratins (k, common impurities observed at low protein levels), and peptides originating from Esp1p (T).

(C) Tandem mass spectrum of the doubly charged ion T_3^{2+} at m/z 407.0. Collisional fragmentation of tryptic peptides produces mainly ions containing the COOH-terminal part of the peptide (Y^n ions). A peptide sequence tag (Mann and Wilm, 1994) was assembled from the mass differences between adjacent Y^n ions (boxed) and used for database searches. A single match, the tryptic Esp1p peptide VILLNIK, was retrieved and confirmed by the complete Y^n ion series. Three other Esp1 peptides were identified: T_1, LPLIR; T_4, ELLESLK (in a mixture with the trypsin autolysis product SISISVAR which has the same nominal mass); and T_5, SLTDLPR. Peptide T_2 was identified by an error tolerant search (Mann and Wilm, 1994) because it is not preceeded by a trypsin cleavage site (K or R) in the Esp1p sequence.

(D) Coimmunoprecipitation of Pds1p with Esp1p. Wild-type and *cdc26Δ* strains containing *PDS1HA6* and *ESP1myc18* were grown at 25°C and then shifted to 36°C for 3 hr. Protein extracts were subjected to immunoprecipitations with an antibody to the myc epitope. Protein extracts and immunoprecipitates were analyzed by immunoblotting with anti-myc, anti-HA, and anti-Clb2p antibodies. +, epitope-tagged allele integrated at the genomic *PDS1* or *ESP1* locus; −, wild-type allele.

within nuclei that lack Pds1p. Subsequently, they proceed with the inactivation of cyclin B/Cdk1 kinases and reembark on a new cell cycle, during which Pds1p reaccumulates. *esp1-1* mutants are therefore not defective in the APC-mediated proteolysis of either Clb2p (Surana et al., 1993) or Pds1p.

esp1-1 pds1Δ Double Mutants Are Inviable

apc mutants fail to separate sister chromatids because they do not degrade Pds1p, whereas *esp1-1* mutants fail to separate sisters despite having destroyed Pds1 protein. This suggests that the lack of sister chromatid separation in *esp1-1* mutants should not be suppressed by deletion of *PDS1*. While trying to test this, we found that *pds1Δ esp1-1* double mutants were inviable, even when spores were germinated at 19°C (data not shown). This suggests that deletion of *PDS1* exacerbates the *esp1-1* defect.

Sister Chromatid Cohesion in *esp1-1* Mutants Depends on Scc1p

To test whether the persistence of sister chromatid cohesion in *esp1-1* mutants depends on Scc1p, we compared the kinetics of sister separation in *esp1-1* single mutants with that in *esp1-1 scc1-73* double mutants. Loss of Scc1 function permitted efficient separation of sister chromatids in *esp1-1* mutants (Figure 5A). To investigate whether Scc1p dissociates from chromosomes in *esp1-1* mutants, we repeated the experiment with an Scc1myc18p strain and followed the association between Scc1myc18p and chromatin using chromosome spreads as unbudded G1 *esp1-1* mutant cells progressed through the cell cycle at 37°C. Scc1p associated with chromatin in late G1 but failed to dissociate following destruction of Pds1p (Figure 5B). Cells accumulated with DNA masses containing a single GFP dot (i.e., paired sisters), and chromatin stained strongly with Cy3 (the marker for Scc1myc18p) (Figure 5C). Similar results were obtained using a version of Scc1p tagged with six HA epitopes (data not shown). We conclude that Esp1p is required for both sister separation and dissociation of Scc1p from chromatin.

Esp1p Localizes to Spindles and Their Poles at the Onset of Anaphase

We modified the endogenous *ESP1* gene to encode a functional fusion protein carrying 18 myc epitopes at its C terminus. Esp1myc18p was distributed throughout

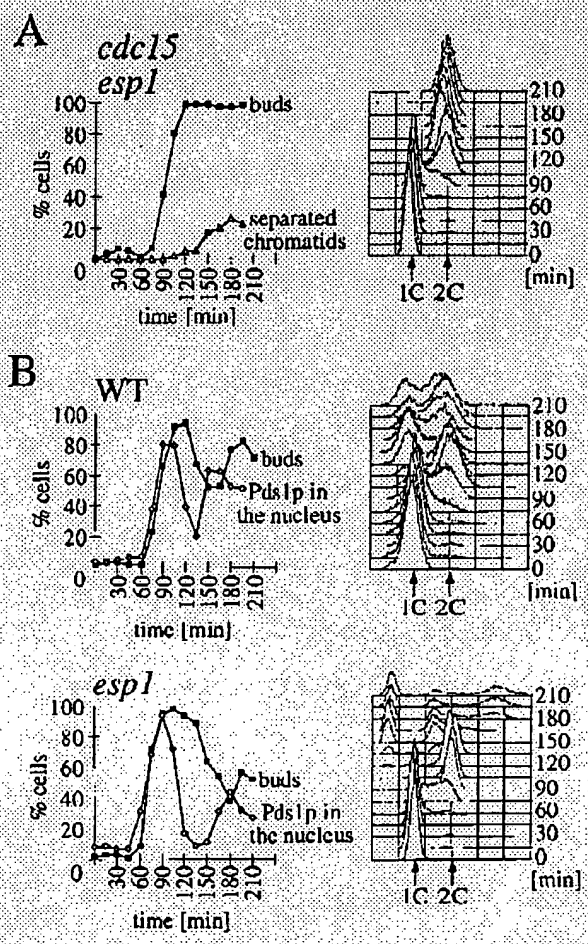


Figure 4. *ESP1* Is Required for Sister Chromatid Separation but Not for Degradation of Pds1p

(A) Lack of sister chromatid separation in *esp1-1* mutant cells. Small G1 cells of an *esp1-1 cdc15-2* strain containing cenV-GFP (K7183) were incubated at 37°C. The control experiment with an *ESP1 cdc15-2* strain is shown in Figure 1.

(B) Degradation of Pds1p does not depend on Esp1 function. Small G1 cells of wild-type (K6803) and *esp1-1* (K6999) strains containing *PDS1myc18* and cenV-GFP were incubated at 37°C. Pds1myc18 protein was detected by indirect immunofluorescence. Only cells completely lacking nuclear Pds1myc18p staining were scored as negative.

the cell during G1, S, G2, and early M phases but was concentrated within dividing nuclei (data not shown). Esp1myc18p's abundance did not vary much during the cell cycle (data not shown). Most nuclear spreads lacked any Esp1myc18p-specific staining, but some contained two large Esp1p-specific dots at opposite ends of the DNA mass. Such nuclei frequently also contained smaller Esp1p-specific dots situated between these two major foci. We never detected any association between Esp1p and bulk chromatin. Most if not all cells with Esp1p dots had two green GFP dots (data not shown), implying that sister kinetochores had separated. The Esp1p-specific dots were seen in *cdc5-1* mutants, which separate sister chromatids but fail to complete anaphase B (Shirayama et al., 1998), but not in *cdc26Δ* mutants or in cells arrested with nocodazole (data not shown). The Esp1p-specific dots at opposite ends of the DNA mass colocalized with tubulin at spindle poles, whereas the Esp1p dots situated midway between these poles were always closely associated with tubulin of the mitotic spindle, at least in those spreads where the latter was visible (Figure 6A). These data suggest that Esp1p associates with mitotic spindles and their poles at the onset of anaphase. Surprisingly, the Esp1p dots associated with spindles, and their poles could not be detected in *pds1Δ* mutants at any temperature, which was not due to lower amounts of Esp1p (data not shown).

Esp1p Promotes Loss of Sister Chromatid Cohesion in the Absence of Mitotic Spindles

The phenotype of *esp1-1* mutants is at least superficially similar to that of *ndc10-1* mutants, which are known to be defective in a centromere binding complex (Doheny et al., 1993). *ndc10-1* mutants also cytokinese and rereplicate their genomes without segregating chromosomes (Goh and Kilmartin, 1993). Furthermore, Esp1p's association with spindles and their poles during anaphase raises the possibility that it might promote sister separation by modulating mitotic spindle activity. To address whether sister separation induced by Esp1p depended on functional kinetochores, we isolated by centrifugal elutriation G1 cells of *ndc10-1* mutants and incubated them at 37°C. In contrast to *esp1-1* mutant cells, sister chromatid separation occcured efficiently in *ndc10-1* mutants, though it was slightly delayed, due possibly to the lack of kinetochore activity (Figure 6B). Sister separation in *ndc10-1* mutant cells even occured in the presence of nocodazole (data not shown) as occurs in *mad* or *bub* mutants (Straight et al., 1996), suggesting that *ndc10-1* mutants are defective in the surveillance mechanism that detects spindle damage and blocks anaphase onset (Murray, 1995). Crucially, loss of cohesion in *ndc10-1* mutants was dependent on Esp1p, because sister chromatids failed to separate in *esp1-1 ndc10-1* double mutants (Figure 6B). Esp1p clearly promotes loss of sister chromatid cohesion by a mechanism that requires neither functional kinetochores nor mitotic spindles.

Overexpression of Esp1p Permits Sister Separation in the Presence of Pds1p

The strict dependence of sister separation on both Esp1p activity and Pds1p destruction suggests that Esp1p might be a sister-separating protein whose activity is blocked by its association with Pds1p. Do cells contain sufficient Pds1p for such a role? Comparisons of the abundance of Pds1myc18p and Esp1myc18p by Western blotting in both cycling cells and *cdc26Δ* mutants arrested at 37°C (data not shown) suggest that, from late G1 till its destruction shortly before anaphase, Pds1p (which is a nuclear protein) is as abundant as Esp1p (which is present both in the nucleus and cytoplasm). These data suggest that there is an excess of Pds1p over Esp1p within nuclei.

To test whether overproduction of Esp1p could titrate out Pds1p and permit cells to separate sisters without Pds1p destruction, we elevated the Esp1p level by integrating three copies of the *GAL1-10* promoter-*ESP1* construct (*GAL-ESP1*) into ts *cdc20-3* mutant cells.

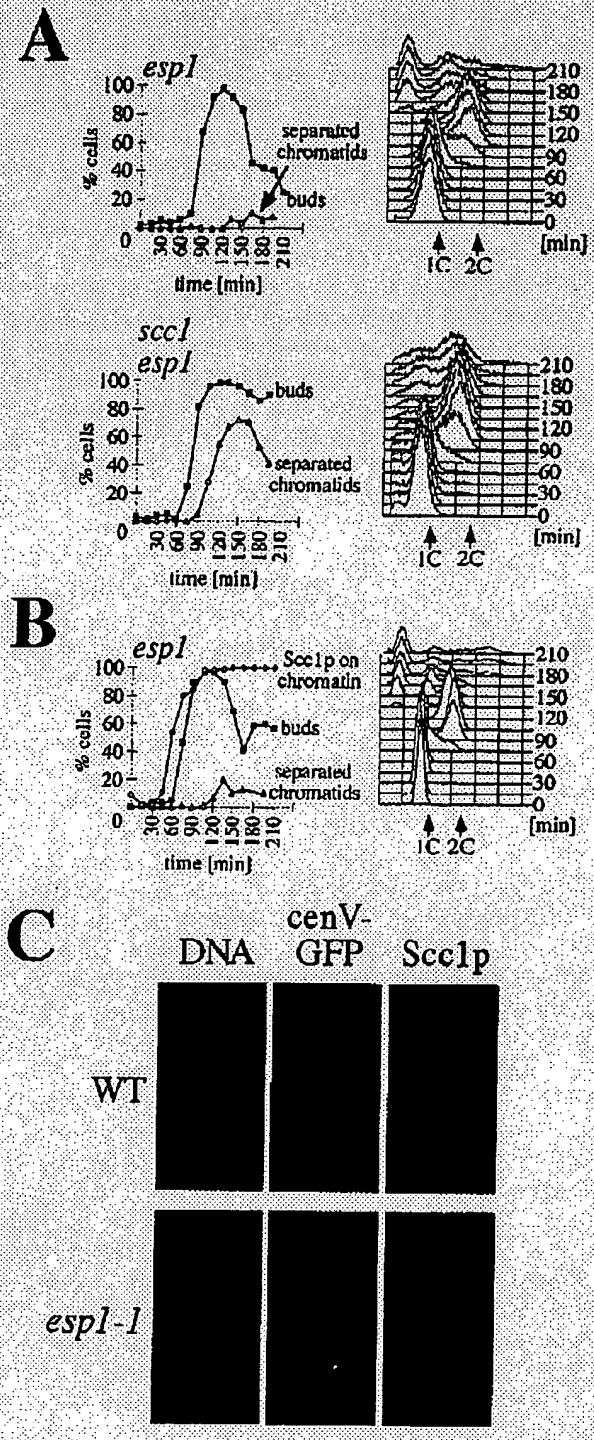


Figure 5. Scc1p Is Required to Maintain Sister Chromatid Cohesion in *esp1-1* Mutants

(A) Sister chromatids separate in *esp1-1 scc1-73* double mutants. Small G1 cells of *esp1-1* (K6842) and *esp1-1 scc1-73* (K6859) strains containing cenV-GFP were incubated at 37°C. Control experiments showing sister separation in wild-type and *scc1-73* strains have already been published (Michaelis et al., 1997).

(B) Esp1p is required for the dissociation of Scc1p from chromatin. Small G1 cells of an *esp1-1 SCC1myc18* cenV-GFP strain (K7030) were incubated at 37°C. Scc1myc18p associated with chromatin was detected by indirect immunofluorescence on chromosome spreads.

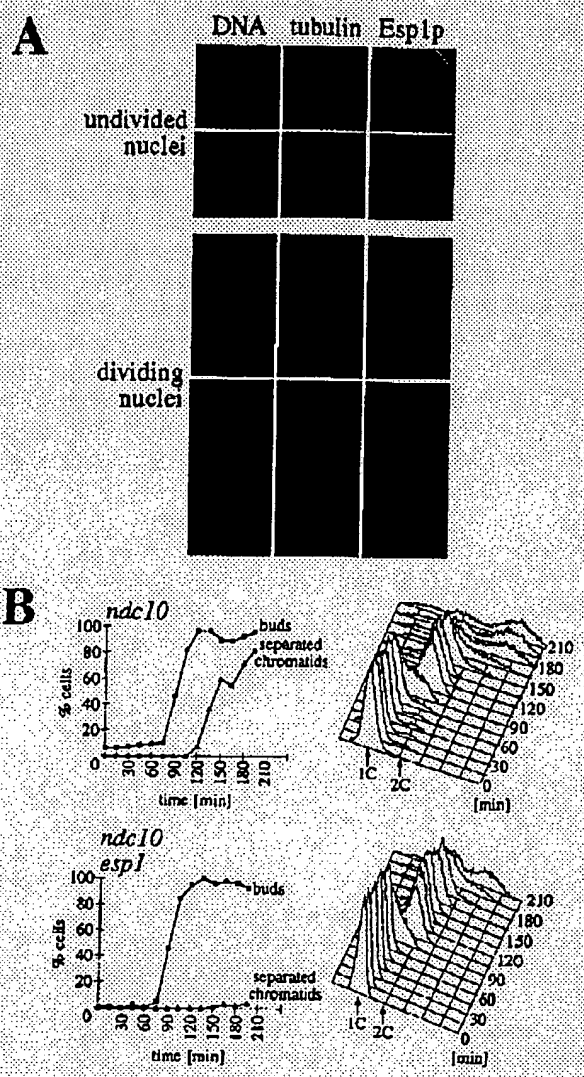


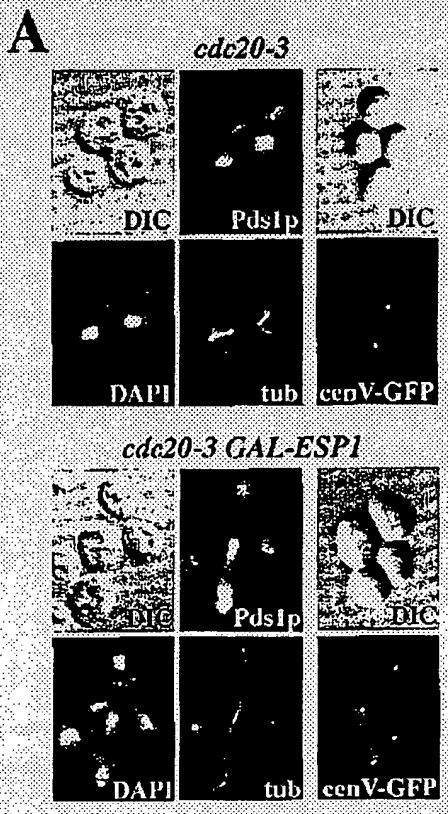
Figure 6. Esp1p Associates with Spindles and Their Poles during Anaphase but Promotes Sister Separation in the Absence of Functional Kinetochores

(A) Chromosome spreads of the *ESP1myc18* cells (K7025) at different stages of the cell cycle. DNA was stained with DAPI. Spindle pole bodies, spindle structures, and Esp1myc18p were detected by indirect immunofluorescence. Two top images, nuclei before and after SPB duplication. Two bottom images, anaphase nuclei with detectable spindles spanning the spindle poles.

(B) Small G1 cells of *ndc10-1* (K6841) and *esp1-1 ndc10-1* (K6884) strains containing cenV-GFP were incubated at 37°C. Cytokinesis takes place in neither strain, so there was no need to use a *cdc15* mutation.

These mutants are defective in the ubiquitination of Pds1p by the APC and separate sister chromatids only if *PDS1* is deleted (Shirayama et al., 1998). Unbudded G1 cells were isolated by elutriation from a culture grown in raffinose and incubated at 37°C in the presence of

(C) Chromosome spreads of cells taken at 135 min. DNA stained with DAPI. The centromeric region of chromosome V visualized by GFP (cenV-GFP). Scc1myc18p associated with chromatin detected by indirect immunofluorescence.



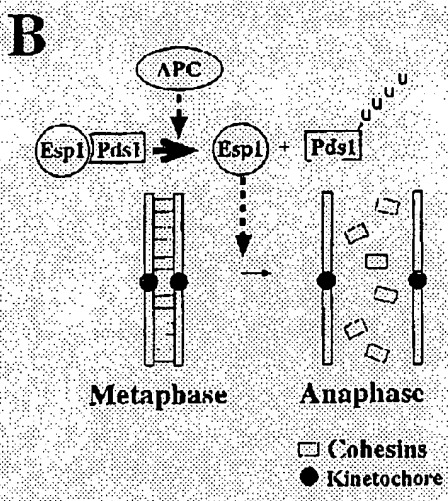


Figure 7. Elevating Esp1p Levels Triggers Anaphase in the Presence of Pds1p as Predicted by the Model

(A) Small G1 cells isolated by elutriation of *cdc20-3* (K7108) or *cdc20-3 GAL-ESP1x3* (K7445) strains containing *PDS1myc18* and cenV-GFP were incubated at 37°C in the presence of galactose. Timing of budding and DNA replication were similar in both strains. All pictures show cells at 165 min. Panels on the left show cells fixed with formaldehyde and processed for indirect immunofluorescence. Panels on the right show cells fixed with ethanol and processed for GFP fluorescence. Pds1myc18p and spindles were detected with antibodies to the myc epitope and tubulin (tub), respectively. Sequences close to cenV were visualized with GFP (cenV-GFP). In wild-type cells sister separation is always preceeded by Pds1p destruction (Michaelis et al., 1997). Overexpression of Esp1p allowed at least 20% of *cdc20-3* mutant cells to completely segregate their genomes in the presence of Pds1p.

galactose. *cdc20-3 ESP1* cells did not segregate their genomes, whereas at least 20% of the *cdc20-3 GAL-ESP1* cells did so within 3 hr. Crucially, the nuclei of *GAL-ESP1* cells that separated sister chromatids in the absence of Cdc20 activity contained high levels of Pds1p (Figure 7A). These cells also contained a fully elongated mitotic spindle stretching between segregated chromosomes (Figure 7A). The presence of a single GFP dot associated with cen V (cenV-GFP) at each end of these "anaphase" cells (Figure 7A) shows that *GAL-ESP1* caused proper disjunction of sister chromatids in the presence of Pds1p. *GAL-ESP1* also permitted sister separation in cells whose Pds1p proteolysis was prevented (at 37°C) by a deletion of *CDC26* (data not shown). We conclude that the dependence of sister separation on APC activity can be bypassed not only by deleting *PDS1* but also by elevating Esp1p levels.

Discussion

The APC is a multisubunit ubiquitin protein ligase necessary for mitotic cyclin proteolysis. The APC is also necessary for sister chromatid separation, even though cyclins themselves need not be destroyed. Sister chromatid separation is therefore thought to depend on ubiquitination by the APC of proteins other than cyclins. One suggestion has been that cohesins holding sister chromatids together might be the key APC targets. Scc1p in yeast is indeed destroyed in an APC-dependent manner, but the timing of this event suggests that it might be a consequence rather than a cause of its dissociation from chromosomes. Pds1p is another candidate; it is degraded shortly before the onset of anaphase and nondegradable versions block sister separation (Cohen-Fix et al., 1996). We have now extended these findings by comparing the kinetics of sister separation in *pds1Δ*, *apc*, and *pds1Δ apc* double mutants. Our finding that inactivation of the APC fails in any way to retard sister separation in mutants lacking Pds1p suggests that the APC might promote sister separation by mediating destruction of Pds1p and no other protein. How then does Pds1p block sister separation? In *apc* mutants, which fail to destroy Pds1p, the cohesin Scc1p remains associated with sister chromatids. We show here that deletion of *PDS1* permits its dissociation with almost wild-type kinetics. The implication is that in *apc* mutants, the persistence of Pds1p alone blocks the dissociation of Scc1p from sister chromatids. One possibility is that Pds1p interacts directly with cohesins and prevents their dissociation from chromosomes. If so, one might expect to find Pds1p bound tightly to chromosomes before the onset of anaphase. We have been unable, however, to demonstrate any association between Pds1p and chromosomes using the same spreading technique used successfully for cohesins. We found

(B) From the end of S phase until the onset of anaphase, sister chromatids are held together by cohesins like Scc1p. During this time, Esp1p is inhibited by its association with Pds1p. Proteolysis of Pds1p, which is mediated by the APC, allows Esp1p to trigger sister separation, possibly by causing dissociation of Scc1p from chromosomes.

Instead that Pds1p is tightly associated with a 180 kDa protein, which was identified by mass spectrometric sequencing as the product of the *ESP1* gene.

We show here that *ESP1*, like the APC, is essential for sister separation and for dissocation of Scc1p from chromosomes. The resemblance between the phenotypes of *esp1* and *apc* mutants ends here. In *apc* mutants Scc1p remains on chromosomes because Pds1p is not destroyed, whereas in *esp1* mutants Scc1p remains on chromosomes despite Pds1p proteolysis occuring normally. Indeed, all cell cycle events except sister separation continue unabated in *esp1-1* mutants, which even undergo cytokinesis and rereplicate their genomes (McGrew et al., 1992). This phenotype contrasts with the metaphase arrest of *apc* mutants and the telophase arrest of *apc pds1*Δ double mutants. These observations suggest that Esp1p has a very specific role in promoting the loss of sister chromatid cohesion and that it is not directly required for any other anaphase process. Because Pds1p inhibits sister separation whereas Esp1p promotes it, we suggest that Pds1p's association with Esp1p inhibits Esp1p's ability to trigger sister chromatid separation (Figure 7B). According to this hypothesis, the APC promotes sister separation only indirectly by allowing the release of Esp1p, a specialized sister-separating protein, from its guardian Pds1p. As predicted by our hypothesis, overproduction of Esp1p permits sister separation in *cdc20* or *cdc26* mutant cells that cannot destroy Pds1p.

How might Esp1p promote loss of sister chromatid cohesion upon its release from Pds1p? We know that Esp1p must cause a global change to chromosomes, because it is needed for the dissociation of Scc1p from a large number of loci throughout the genome. We also know that Esp1p causes loss of cohesion even in the absence of any connection between centromeres and microtubules, because sister separation occurs in an Esp1p-dependent manner in *ndc10-1* mutants, even in the prasence of nocodazole. The splitting of acentric chromosome fragments obtained by laser microsurgery at the same time as intact chromosomes is also consistent with such a notion (Liang et al., 1993).

We find Esp1p distributed throughout the cell from late G1 until metaphase. It is neither concentrated in nor excluded from the nucleus. Pds1p in contrast is concentrated within the nucleus during this period (Cohen-Fix et al., 1996). At the metaphase to anaphase transition, Esp1p concentrates within nuclei, and a fraction of it associates with spindles and their poles. The latter depends on the APC and presumably occurs as a consequence of Pds1p proteolysis. More surprisingly, it also depends on the prior presence of Pds1p. We saw little or no association of Esp1p with spindles and their poles in *pds1*Δ mutants, even when they separate sisters on time when grown at their permissive temperature, which casts doubt as to whether Esp1p's assocation with spindles and their poles is relevant to its role in promoting sister chromatid separation.

Only a fraction of Esp1 molecules associate with spindles and their poles during anaphase, and the free Esp1 molecules that are not associated with spindles might be those actually responsible for loss of sister cohesion. Esp1p might, for instance, interact transiently with cohesins and facilitate their dissocation from chromosomes. Alternatively, it might destroy cohesion by an indirect mechanism, by generating a global change within nuclei that is more directly responsible for weakening sister chromatid cohesion. A candidate would be the concentration of Ca^{2+}, which appears to change at the metaphase to anaphase transition (Groigno and Whitaker, 1998).

If loss of sister chromatid cohesion were solely regulated by APC-mediated removal of Pds1p from Esp1p/Pds1p complexes, then one would expect sister separation to occur prematurely in *pds1*Δ mutants, which could be a lethal event. However, this is not the case. In cells growing at low temperatures (e.g., 25°C), *PDS1* is neither necessary for proliferation (Yamamoto et al., 1996a) nor for preventing premature separation of sister chromatids (G. Alexandru, personal communication). The implication is that the putative inactivation of Esp1p by the binding of Pds1p cannot be the only mechanism for regulating sister chromatid separation. In addition to its control by an unstable inhibitor, Esp1p may be subject to other controls, such as phosphorylation by mitotic kinases, for example Cdks, which ensure that Esp1p is only active once cells have aligned sister chromatids on the mitotic spindle. The separation of sister chromatids is one of the most important transitions in the cell cycle, and it comes as no surprise that this event is regulated by more than one mechanism. Destruction of Pds1p might merely "unlock the door," which must nevertheless still be "opened."

The lethality of *pds1*Δ mutants at 37°C is not due to premature separation of their sister chromatids (Figure 1). Instead, there are four reasons for believing that *pds1*Δ lethality might be due to a reduction in Esp1p activity. First, Esp1p's association with spindles and their poles is greatly reduced by a deletion of *PDS1*. Second, *pds1*Δ *esp1-1* double mutants are inviable. Third, the lethality of *pds1*Δ mutants at high temperatures is suppressed by multicopy plasmids containing the *ESP1* gene or by *GAL-ESP1* (data not shown; B. Byers, personal communication). Fourth, *pds1*Δ mutants separate sister chromatids inefficiently at 37°C. This suggests that Esp1p's guardian, Pds1p, regulates Esp1p's activity both negatively and positively. For example, Esp1p might only be fully active when released (by the APC) from its complex with Pds1p.

In addition to controls that operate in cycling cells, sister separation is blocked by surveillance mechanisms (checkpoints) that detect damage to DNA or to spindles. A key question is whether such checkpoints block sister separation solely by blocking the proteolysis of Pds1p or whether they also act on components such as Esp1p. *pds1*Δ mutants are at least partially defective in blocking sister chromatid separation in the presence of damaged DNA or microtubules, suggesting that at least some surveillance mechanisms work by regulating APC-mediated proteolysis (Yamamoto et al., 1996b).

Esp1p is homologous to Cut1p from *Schizosaccharomyces pombe* (Uzawa et al., 1990) and to BimB in *Aspergillus nidulans* (May et al., 1992), both of which are also required for nuclear division but not for reentry into the next cell cycle. The homology between these proteins is largely confined to their C-terminal 300 amino acids,

which are similarly conserved in potential homologs from humans and *Caenorhabditis elegans*. *cut1* mutants manage to separate chromatids at centromeric regions but do not segregate sister chromatids fully from each other (Funabiki et al., 1993). We suggest that these *cut1* alleles are "leaky" and that Cut1p is not merely needed for the proper segregation of chromatids but is required like Esp1p to initiate sister chromatid separation. Thus, the Esp1/Cut1 class of sister-separating proteins might be called "separins."

Cut1p binds to Cut2p, a protein with Pds1-like properties, and is associated with mitotic spindles (Uzawa et al., 1990; Funabiki et al., 1993, 1996a). Though Cut1p and Esp1p are clearly related proteins with similar functions, there is little obvious sequence similarity between Pds1p and Cut2p. Furthermore, *cut2*$^{+}$, unlike *PDS1*, is an essential gene and is necessary for chromosome segregation (Funabiki et al., 1996a). Despite these differences, we suspect that Cut1p and Cut2p in *S. pombe* perform similar functions to Esp1p and Pds1p in *S. cerevisiae*. We suggest that during S, G2, and early M phase, Esp1p's "guardian" Pds1p not only inhibits Esp1p activity but also prepares it for activation by as yet unkown cell cycle signals. This "helpful" role is important but not essential for *S. cerevisiae*, except at 37°C. We propose that Cut2p performs both roles for Cut1p, with the difference that Cut1p's prior association with Cut2p is not merely helpful but actually essential for Cut1p function. The lack of any striking homology between Pds1p and Cut2p may stem from the fact that Pds1p and Cut2p are primarily inhibitors of Esp1/Cut1-like proteins and do not themselves have a conserved role in the enzymology of loosening cohesion between sisters. Inhibitors of S phase Cdks, whose proteolysis has an important role in promoting S phase, are likewise not highly conserved. If we are correct in thinking that Cut1/Cut2 complexes are mechanistically equivalent to Esp1/Pds1 complexes, then the existence of animal homologs of Esp1- and Cut1-like proteins make it seem likely that similar complexes will also regulate the onset of anaphase in humans.

Experimental Procedures

Yeast Strains and Growth Conditions

All strains were derivatives of W303. YEP medium (Rose et al., 1990) was supplemented with either 2% raffinose (YEPraf) or 2% glucose (YEPD). To obtain synchronous cultures, cells were grown in YEPraf medium at 25°C (or at 19°C in case of *pds1Δ* strains), and small, unbudded G1 cells were isolated by centrifugal elutriation (Schwob and Nasmyth, 1993). These cells were then inoculated into YEPD medium to a density of 6×10^5 cells per ml, usually at 37°C. Chromosomes were visualized by a tet repressor-GFP fusion protein (tetR-GFP) binding to an array of tet operators (tetOs) integrated at either the *ura3* locus near the centromere of chromosome V (Michaelis et al., 1997, referred to as cenV-GFP) or at the *BMH1* locus 30 kbp from the right end of chromosome V (telV-GFP).

Strain Constructions

To tag the *ESP1* gene, a YIplac204 (Gietz and Sugino, 1988) based tagging plasmid was constructed, which contained a fragment spanning the C-terminal part of *ESP1* and a part of its 3′UTR, with an SpeI site created in front of the stop codon. Two SpeI cassettes containing 9 myc epitopes each (Michaelis et al., 1997) were cloned into the SpeI site. The resulting plasmid was linearized with ClaI and integrated into the yeast genome at the endogenous *ESP1* locus. The *ESP1myc18* strain (K7025) grew normally at 37°C, demonstrating that the epitope-tagged version is functional. To overproduce the *ESP1* gene, a YIplac204-based plasmid was constructed that contained *ESP1* coding sequence with 1 kb of its 3′UTR with a HindIII site created before the start codon, to which the *GAL1-10* promoter was attached. The resulting plasmid was linearized with BstXI and integrated into the yeast genome at the *trp1* locus. For further studies we chose a triple integrant of the *GAL-ESP1* construct. A *PDS1HA6::HISMX* allele was constructed by PCR-mediated epitope tagging of the genomic *PDS1* locus. *PDS1HA6* strains grew normally at 37°C.

Mass Spectrometric Identification of Esp1p

Immunoprecipitations from extracts of metabolically labeled cells were carried out as described (Zachariae et al., 1996b). For mass spectrometric analysis, the Pds1myc18p-p180 complex was purified by one-step immunoprecipitation from an unfractionated whole-cell extract prepared from *PDS1myc18 Δpep4* cells (1.0×10^{10}) using the procedure of Zachariae et al. (1998). The 180 kDa band was excised from a single one-dimensional gel stained with silver and digested in-gel with trypsin (Shevchenko et al., 1996). The recovered unseparated peptide mixture was analyzed by tandem mass spectrometric sequencing as described (Wilm et al., 1996b) using an API III triple quadrupole mass spectrometer (Perkin Elmer-Sciex, Ontario, Canada) equipped with the nano-electrospray ion source (Wilm and Mann, 1996a). The gel actually used to identify Esp1p is shown in Figure 1A of Zachariae et al. (1998). For immunoprecipitation-immunoblotting experiments (Figure 3D), 1.5×10^8 cells were broken in 0.4 ml buffer B70. Extracts (0.33 ml, 6 mg) were incubated with 0.1 ml protein A-Sepharose and then with 0.033 ml protein A-Sepharose carrying the anti-myc antibody 9E10. Preparation of extracts and immunoprecipitations were carried out essentially as described previously (Zachariae et al., 1998). Extracts and immunoprecipitates were separated on 0.8 mm, 7.5% SDS-polyacrylamide gels and analyzed by immunoblotting.

Other Techniques

A FACScan (Becton-Dickinson) was used for flow cytometric analysis of cellular DNA content as described (Epstein and Cross, 1992). Chromosome spreading and visualisation of yeast chromosomes using the tetR-GFP/tetO system were performed as described previously (Michaelis et al., 1997). Cells were prepared for indirect immunofluorescence according to Piatti et al. (1996). Myc-tagged proteins were detected with the 9E10 monoclonal antibody and a CY3-conjugated secondary antibody. Spindles were detected with a rabbit antiserum to yeast tubulin and a fluorescein isothiocyanate-conjugated secondary antibody. Pictures were taken with a Quantix charge-coupled device camera (Photometrics) mounted on a Zeiss Axioplan 2 microscope.

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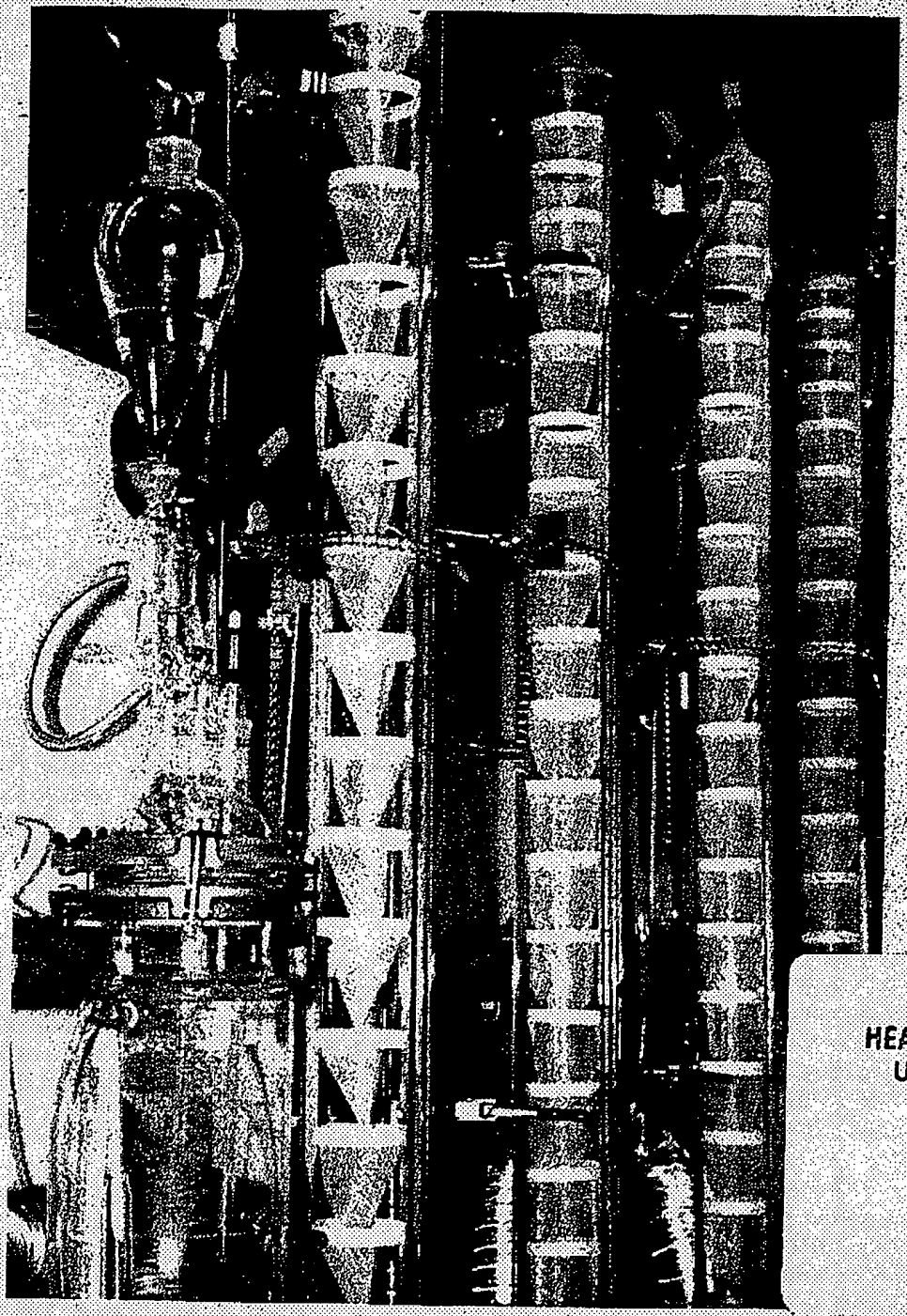

Ethanol Intoxication in *Drosophila*

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Synthesis of Multiple Peptides on Plastic Pins

Scanning protein sequences by bioassay for smaller bioactive peptide sequences requires a source of many peptides homologous with the parent protein sequence. This unit deals with one of the synthetic methods for making such sets of peptides (see Fig. 18.2.1). The key to preparing large numbers (hundreds to thousands) of synthetic peptides in a short time and at minimal cost is to use a parallel synthesis technique which is efficient and can be done on a small scale. The multipin technology is suitable because it can be performed without expensive synthesizers and it uses equipment available to most laboratories. Prior experience with organic synthesis techniques or peptide chemistry is useful but not essential. The products of synthesis by multipin technology are unpurified peptides which are useful as screening reagents and may also be used to prepare purified peptide on a small scale.

Most multipin techniques exploit the conventional 8 × 12 matrix layout of common microtiter equipment which simplifies handling of the synthesis, the products (peptides), and the test results. Computer assistance with synthesis and data analysis also speeds the cycle from designing the experiment through analyzing the results.

With multipin technology, peptides are synthesized in parallel on plastic "pins" (Fig. 18.2.2) to give sets of peptides suitable not only for B and T cell epitope scanning but also for other bioassays. Peptides can be either permanently bound to the surface of the plastic for direct binding assays, or they can be released into solution. There is a choice of N- and C-terminal peptide endings. For solution-phase peptides, the synthesis scale can be 1 or 5 μmol (for a 10-mer, ~1 mg or 5 mg, respectively). The preferred coupling/deprotection chemistry used is the milder 9-fluorenylmethyloxycarbonyl (Fmoc) protection scheme rather than the older *t*-butyloxycarbonyl (*t*-Boc) protection scheme (see *UNIT 18.1*), thus reducing the level of chemical safety risk arising from synthetic peptide chemistry.

This unit covers the strategy of the multiple peptide approach to biological scanning, the synthetic protocols, and the handling of peptides after synthesis—cleavage, preliminary purification, storage, and analysis (see Basic Protocol). It is specific for the multipin technique using equipment obtained from Chiron Technologies, although some of the approaches are applicable to other multiple synthesis techniques. Procedures for multipin equipment obtained from other suppliers may differ from the procedures described here, and the manufacturer's literature should be consulted. This unit also includes protocols for preparing Fmoc–amino acid solutions (see Support Protocol 1) and for acetylating (see Support Protocol 2) or biotinylating (see Support Protocol 3) synthesized peptides.

STRATEGIC PLANNING

For a protein whose primary structure is known, the conceptually simplest method of locating all the bioactive linear peptide sequences is to make all possible peptide subsets of the protein sequence and test them. If only selected parts of the sequence are synthesized, or only the predicted active parts, bioactive sequences could be missed. The use of a set of highly overlapping peptides likewise reduces the possibility that the most bioactive sequences might be missed because they are absent from the set. A set of all overlapping 20-mers offset along the sequence by one residue at a time should capture the entire set of, for example, helper T cell epitopes, and this is a much more reliable approach than trying to predict motifs. In reality, a synthetic peptide scan through a protein is a compromise between the cost and effort in making and screening all peptides and the

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need for completeness. Thus, one worker may choose to make all overlapping 8-mers to find the linear (continuous) B cell epitopes, and another may make 12-mers offset along the sequence by five residues for the same purpose. In each case, all sequences of eight residues from the protein are present in at least one peptide, but the latter approach requires only one-fifth the number of peptides.

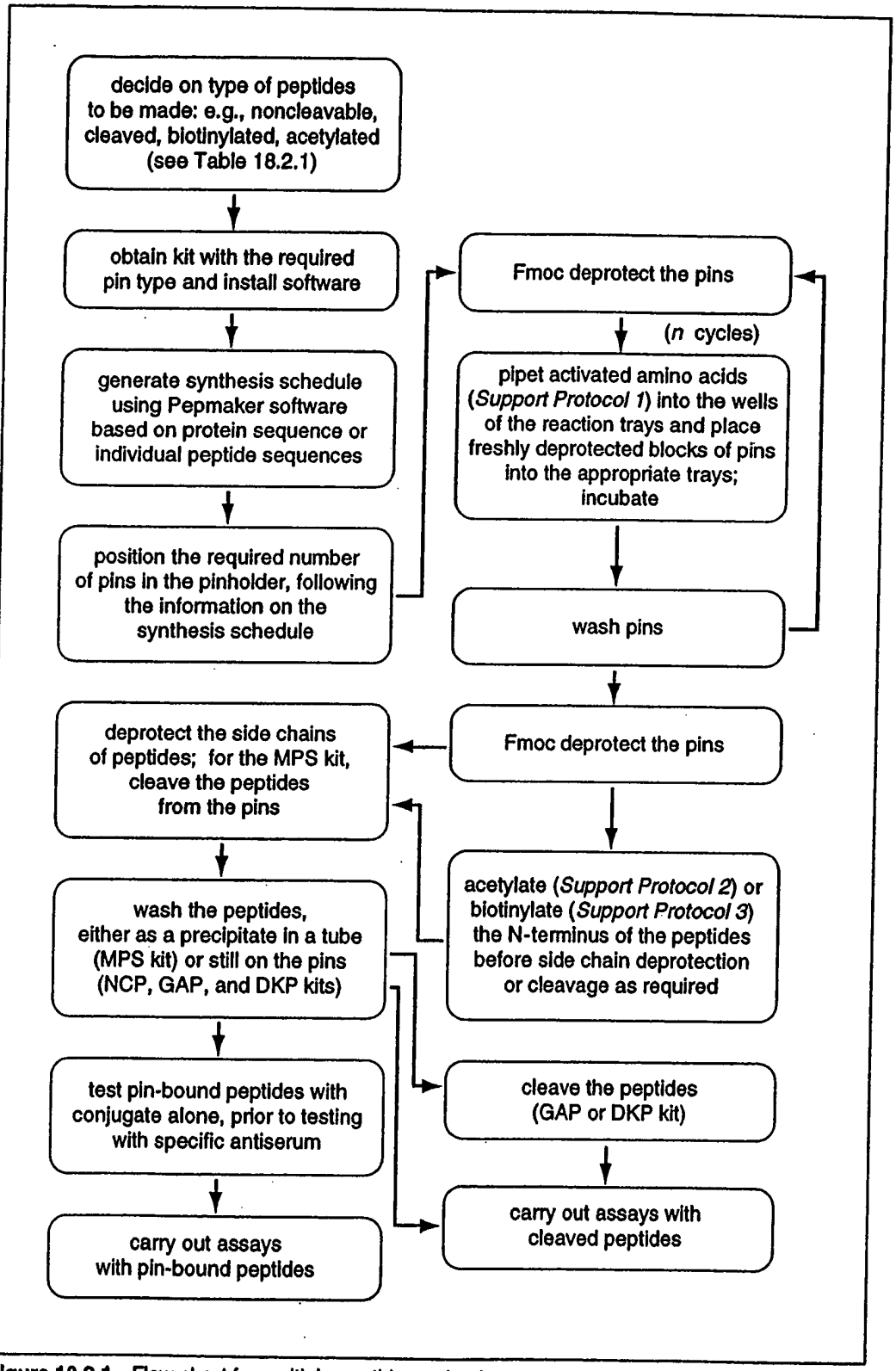

Figure 18.2.1 Flow chart for multipin peptide synthesis.

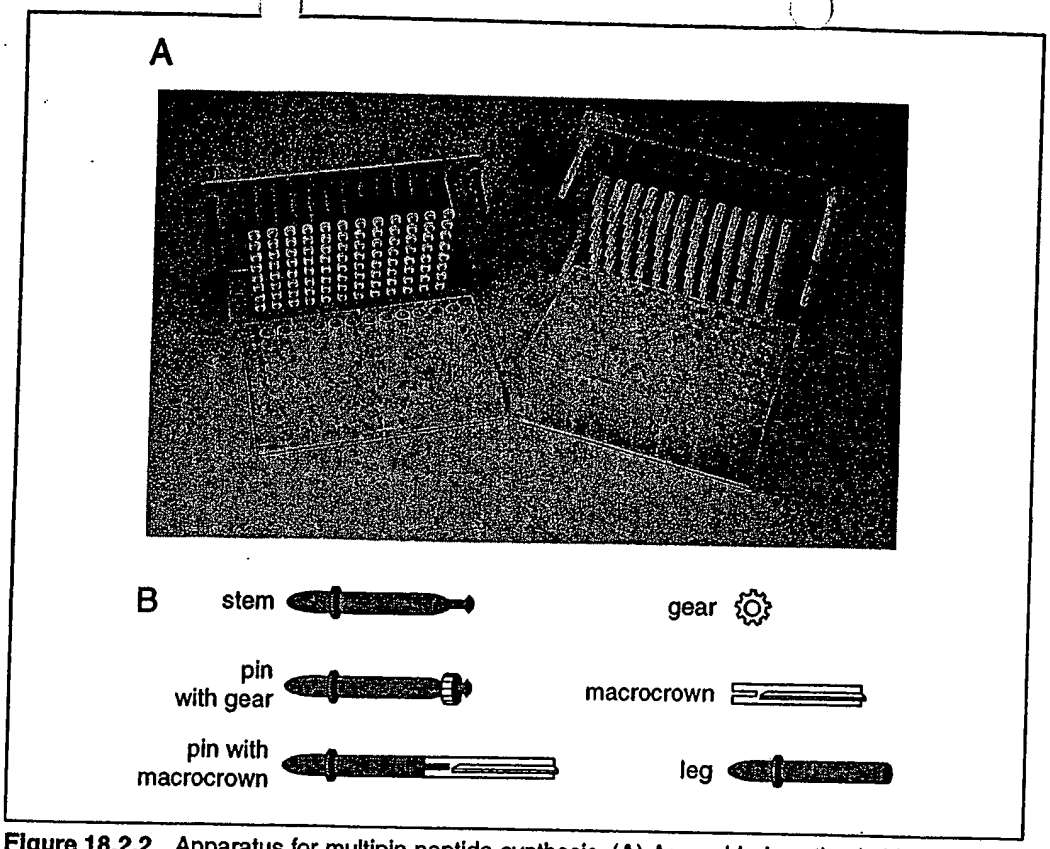

Figure 18.2.2 Apparatus for multipin peptide synthesis. (**A**) Assembled synthesis block with 96 gears (left) or 96 macrocrowns (right). (**B**) Components of the pin assembly. Components are either push-fitted together (e.g., legs or stems into the pin holder) or clipped on (gears or macrocrowns onto stems). All components are solvent-resistant plastic, either polyethylene, polypropylene, or copolymers of these two monomer types.

Planning the Synthesis

Synthetic peptides are assembled by solid-phase synthesis one amino acid at a time, commencing with the C-terminal end of the peptide on the solid phase (see *UNIT 18.1*).

The assembly process, or coupling, requires activation of the α-carboxyl group of each incoming amino acid to make it reactive with the α-amino group of the growing peptide chain. To prevent unwanted polymerization or side reaction, reactive groups in each amino acid must be temporarily protected, and the protecting group removed before further reaction can be carried out. The protecting group on the α-amino function of the most recently added amino acid must be removed before another amino acid can be coupled to it, so the α-amino protection must be labile under conditions that do not remove side-chain protection. Later, the side-chain-protecting groups must be removable under conditions that do not attack the peptide bonds. The two common protecting group "schemes" are known as *t*-butoxycarbonyl (*t*-Boc) or 9-fluorenylmethyloxycarbonyl (Fmoc). The protecting group scheme currently recommended for multipin peptide synthesis is the milder Fmoc scheme, which is the only scheme described in this chapter.

Before beginning to plan the actual synthesis in detail, a choice needs to be made regarding how the peptides will eventually be presented in the bioassay. The options available to investigators are listed in Table 18.2.1.

For noncleavable peptide (NCP) kits, peptides are permanently bound on the solid phase (pin surface) and can be used for direct binding assays but not for interaction with living cells or other complex (e.g., multicomponent) systems. In this case, the peptides must be

Table 18.2.1 Types of Pins for Multipin Peptide Synthesis[a]

Name	Linker[b]	Physical format[c]	Loading	Final form of peptide
NCP	Noncleavable	Gear	50 nmol	(N-capping)-PEPTIDE-linker-pin
MPS	AA ester	Macrocrown	5 μmol	(N-capping)-PEPTIDE-acid
MPS	Rink amide	Macrocrown	5 μmol	(N-capping)-PEPTIDE-amide
DKP	DKP-forming	Gear	1 μmol	(N-capping)-PEPTIDE-DKP
GAP	Glycine ester	Gear	1 μmol	(N-capping)-PEPTIDE-glycine-acid

[a]Abbreviations: DKP, diketopiperazine; GAP, glycine acid peptide; MPS, multiple peptide synthesis; NCP, noncleavable peptide; (N-capping), a free amine, acetyl group, or biotin; PEPTIDE, the sequence of the peptide being made.

[b]Nature of linker between peptide and graft polymer on the pin: noncleavable linker, β-alanine-hexamethylenediamine; DKP, diketopiperazine; AA ester, amino acid ester; Rink amide, Rink amide–forming linker.

[c]See Figure 18.2.2B.

"regenerated" between repeat assays by disrupting the peptide-ligand interaction without damaging the peptide. The quantity of peptide made is very small (50 nmol), but it is sufficient to provide a high surface density of peptide for direct binding assays.

In the other options, peptides are synthesized on pins and then released into solution. The mechanism of peptide release into solution affects the postsynthesis handling and thus the suitability of peptides produced by each cleavage method for various assay systems.

For multiple peptide synthesis (MPS) kits, the released peptides have a "native" free acid or an amide carboxy terminus. To make free acid C-termini, it is necessary to use macrocrowns that already have the first (C-terminal) amino acid on them because the chemistry of forming the first (ester) link is too difficult for the inexperienced user. In contrast, the Rink amide linker allows formation of a peptide with a C-terminal amide of any amino acid by adding the C-terminal amino acid to the Rink handle macrocrown using the standard amino acid coupling protocol. A Rink amide linker is a linker that can accept an amino acid but then can be cleaved in trifluoroacetic acid (TFA) to release the amide form of that amino acid (Rink, 1987). Although acid or amine endings are often the most desirable peptide format to have, they are also the most complex to produce because the cleavage of the peptides from the pin is into neat TFA plus scavengers which needs to be evaporated to recover the peptide. The scale of peptide synthesis for MPS kits is 5 μmol (~5 mg of a decamer).

For glycine acid peptide (GAP) kits, peptides with a glycine at the carboxy terminus are cleaved as the free acid, so that the C-terminal residue is a natural amino acid (glycine) and is not blocked. The peptides are also relatively simple to release from the pin and require little postsynthesis handling. However, the presence of glycine at the C-terminus may be undesirable where the C-terminus plays an important role in peptide bioactivity. The scale of synthesis for GAP kits is 1 μmol (~1 mg of a decamer).

In diketopiperazine (DKP) kits, peptides are synthesized with a DKP group at the C terminus. The DKP group is a cyclic dipeptide formed from C-terminal lysine and proline residues during the facile cleavage of the peptide under the mildest possible conditions: neutral aqueous buffer. In applications where the presence of the DKP group is acceptable, this type of peptide can make the downstream processing of synthetic peptides very simple and fast. The peptides can be placed into a bioassay system immediately after completing the cleavage. The scale of synthesis for DKP kits is 1 μmol (~1 mg of a decamer).

For these five kit options, it is also possible to choose a variety of N-terminal endings on the peptides. For example, it may be desirable to acetylate pin-bound peptides (see Support Protocol 2) to eliminate the positive charge that would otherwise be present on

the α-amino group of the N-terminal residue, or to enhance the activity of a peptide in a T helper assay (Mutch et al., 1991). A handy option for cleaved peptides is to place a biotin group on the N-terminus (see Support Protocol 3) so the peptide can be captured using avidin or streptavidin. These additions must be made prior to side-chain deprotection of the peptides.

There are other configurations for multiple peptide synthesis—e.g., the SPOTS or "peptides on paper" system (Zeneca/CRB), the RaMPS system (DuPont), and multi-synthesizer machines (e.g., Advanced ChemTech).

Assessing Peptide Sequences

Peptides differ so much in properties that it is important to assess the likely properties of the peptides before attempting to synthesize them. Peptide length and hydrophobicity are the two main attributes affecting successful synthesis. The longer the peptide, the lower will be the purity of the product, as each amino acid coupling cycle is never 100% efficient. Synthesis of peptides longer than 20 residues should be avoided unless special attention can be given to each sequence. Hydrophobic peptides may be difficult to synthesize, but more significantly they may be poorly soluble in aqueous buffers, restricting their ultimate usefulness in bioassays. Prior to beginning synthesis of a set of peptides, it is sensible to assess them all for hydrophobicity (Fauchere and Pliska, 1983; *UNIT 2.2*) and decide if all should be attempted as they stand. In many cases, it is possible to choose slightly different peptides (longer, shorter, or using a different starting and finishing point in the homologous protein sequence) that will have more user-friendly properties.

As well as these general factors affecting peptides, particular peptide sequences may have characteristics that make them difficult to synthesize, or they may be problematic after synthesis. It is not feasible to discuss all the common problems here. To help assessment of peptide sequences, a software application called Pinsoft is available free from Chiron Technologies. This allows any sequence to be typed in, and an assessment is automatically reported.

Generating Peptide Sequences

Computer software (Pepmaker) supplied with synthesis kits allows sets of overlapping peptide sequences to be generated from a protein sequence computer file using the single-letter amino acid code. Alternatively, sequences can be created using a word processor and the resulting computer text file can then be used by Pepmaker to guide synthesis. The use of this software simplifies the otherwise complex and tedious task of adding the right amino acids to each reaction plate on each synthesis cycle.

MULTIPIN SYNTHESIS OF PEPTIDES

BASIC PROTOCOL

Derivatized pins with macrocrowns or gears are attached to a pin holder. Each peptide is built up on the reactive surface of one pin by successive cycles of amino acid coupling, followed by washing and removal of the 9-fluorenylmethyloxycarbonyl (Fmoc) amino-protecting group to prepare for the next amino acid coupling cycle. A critical step is properly dispensing activated amino acid solutions into the appropriate wells of each reaction tray. A list of the well locations for dispensing of each amino acid is generated by the Pepmaker software for this purpose. When the peptides are complete, trifluoroacetic acid (TFA) that contains scavengers is used to remove the side-chain-protecting groups, and for MPS kits, to cleave the peptides from the pins. The manual provided with each type of kit (see Table 18.2.1) includes instructions and hints for kit-specific procedures.

NOTE: All reagents should be of the highest grade possible, preferably peptide synthesis or analytical reagent grade.

Materials

20% (v/v) piperidine/dimethylformamide (DMF; see recipe)
DMF, analytical reagent grade
Methanol, analytical reagent grade
100 mM activated 9-fluorenylmethyloxycarbonyl (Fmoc)–protected amino acid solutions (see Support Protocol 1)
Side chain deprotecting (SCD) solution (see recipe)
Acidified methanol: 0.5% (v/v) glacial acetic acid/methanol
1:2:0.003 (v/v/v) ether/petroleum ether/2-mercaptoethanol (2-ME)
1:2 (v/v) ether/petroleum ether
0.1 M NaOH
0.1 M acetic acid
0.1 M sodium phosphate buffer, pH 8.0 (*APPENDIX 2E*)
Sonication buffer (see recipe)

Peptide Synthesis Starter Kit (e.g., Chiron Technologies) of the desired type, containing:
  Pepmaker computer program and ELISA reading and plotting programs
  Manual
  Pins with gears or macrocrowns
Storage boxes or sealable bags, polyethylene or polypropylene (ICN Biomedicals)
Pipettor tips, polyethylene or polypropylene (ICN Biomedicals)
0.3- or 1.5-ml reaction trays, polyethylene or polypropylene (Chiron Technologies, Nunc, or Beckman)
Sonicator with power output of ~500 W
Dry nitrogen
Rack containing 96 1-ml polypropylene tubes (Bio-Rad)
10-ml capped conical polypropylene centrifuge tubes

Additional reagents and equipment for N-terminal acetylation (see Support Protocol 2; optional) or biotinylation (see Support Protocol 3; optional)

CAUTION: Perform all chemistry steps in a well-functioning chemical fume hood. Wear solvent-resistant gloves, safety glasses, and protective clothing. The reagents can be flammable, toxic, and/or carcinogenic. Avoid sources of contamination which may affect the pins, including direct contact with the bench surface or exposure to vapors. The reagents for multipin synthesis can be handled in unsealed systems, but the amount of time that these reagents are left exposed to the open air should be minimized by using capped containers for liquids or polyethylene bags for pins wherever practical. Local regulations for safe disposal of solvents and used reagents must be followed.

Prepare synthesis schedule and equipment

1. Use the Pepmaker computer program according to the instructions to generate the required set of peptide sequences (Fig. 18.2.3). Generate the printouts, which show for each coupling cycle how much of each amino acid solution, catalyst, and activating agent needs to be prepared (see Fig. 18.2.4) and where each amino acid solution is to be added to the reaction tray (see Fig. 18.2.5).

   The standard microtiter plate layout is an 8 × 12 matrix, in which the eight rows are identified as A through H and the twelve columns are identified as 1 through 12. However, the Pepmaker software uses a designation in which the column number is given first followed by a number designation for the row, beginning with row H, given in parentheses—i.e., 1(1) for well H1, 1(2) for well G1, 2(1) for well H2, and 12(8) for well A12 (see Fig. 18.2.6).

2. Label each pin holder block indelibly on the top (e.g., Synthesis #1, Block A, Synthesis #1, Block B, and so forth), preferably by scratching into the plastic with a sharp tool. Place the label where it will help orient the block so that the pins are not accidentally placed into amino acid solutions in an inverted orientation. For example, keep pin H1 and well H1 at the lower left corner of the block (Fig. 18.2.6).

Ink labels will run or disappear with exposure to solvents.

The multipin system is based on the standard microtiter plate layout. The block is the complete unit and consists of the pin holder, which is the support that holds 96 pins (in an 8 × 12 array with standard ELISA microtiter plate spacing), and five legs to support the device and correctly position the active surfaces. A pin consists of an inert stem that supports either a gear or a macrocrown, both of which have an active surface on which the peptide is synthesized (see Figure 18.2.2). A gear is a detachable gear-shaped unit that fits on the thin end of a stem. A macrocrown is a detachable, vaned tip that fits on the thin end of a stem. It is made of high-density polyethylene and the surface is derivatized to give a solvent-compatible polymer matrix. Macrocrowns are provided in two forms: one has a linker that cleaves to give peptides with an amide at the carboxy terminus; the other has a linker that cleaves to give the free acid at the carboxy terminus and is supplied with an amino acid already attached to the linker. The reaction tray used for the synthesis is a polyethylene or polypropylene tray consisting of 96 wells in the standard microtiter plate 8 × 12 matrix. Shallow reaction trays (0.3-ml) are used with gears and deep trays (1.5-ml) are used with macrocrowns.

3. Remove any pins that are not required for the first cycle of amino acid coupling and store them dry in a plastic bag in the refrigerator until needed.

Some pins need to be removed when the peptides in the synthesis are of various lengths because the software is designed to arrange all peptides to complete their synthesis on the same (final) coupling cycle. This approach eliminates unnecessary Fmoc–deprotection cycles for pins that are designated to carry the shorter peptides. The synthesis printout from the Pepmaker software shows which pins need to be added for each cycle of amino acid addition (Fig. 18.2.5). Pins (stems) can be pushed out from the top side of the pin holder. In the case of the MPS kit, where the first amino acid is already on the macrocrown as supplied, choose and mount the correct macrocrown for each position on the block.

Deprotect α-amino groups

4. Add 20% piperidine/DMF to a bath and place the pins in the bath so that the tips (macrocrowns or gears) are covered. Cover and let stand for 20 min at room temperature.

CAUTION: Piperidine is flammable.

The volume of reagent needed for all the bath steps depends on the shape of the bath, the critical factor being that all surfaces of the pins (i.e., the gears or macrocrowns) bearing the peptide need to be totally covered. A small bath suitable for gears is the upturned polypropylene lid of a pipettor tip box. For macrocrowns, deeper baths or deep-well polypropylene trays as supplied with the kit can be used.

5. Remove the block from the bath, shake off the excess liquid, and then wash the pins in a DMF bath for 2 min at room temperature.

Again, the DMF must fully cover the tips.

6. Shake off the excess DMF and immerse the block completely in a deep bath of methanol for 2 min so that all surfaces of the block are washed.

CAUTION: Methanol is flammable and toxic.

In a shallower bath the block can be turned over so that the pin holder part is washed as well.

A

```
GENERAL NET SYNTHESIS SCHEDULE NUMBER : 1          Page 1
 Description: Example of a scan through Sperm Whale Myoglobin
        8-mer peptides based on the sequence MBN-SW
              Peptide spacing increment is 1
     Segment  1:  146 peptides starting at residue 1
          First peptide: [VLSEGEWQ]
          Last  peptide: [YKELGYQG]
          Protein sequence: MBN-SW (153 residues)
   1: VLSEGEWQLV  LHVWAKVEAD  VAGHGQDILI  RLFKSHPETL  EKFDRFKHLK
  51: TEAEMKASED  LKKHGVTVLT  ALGAILKKKG  HHEAELKPLA  QSHATKHKIP
 101: IKYLEFISEA  IIHVLHSRHP  GNFGADAQGA  MNKALELFRK  DIAAKYKELG
 151: YQG
              Amino Acid set to be used - AASET1
   aaset 1:Free acid L-Fmoc amino acids - DIC/HOBt chemistry
 Number of copies of each peptide  1
 Schedule based on a 250 microliter fill/well
   (Well concentration is 100 mM)
```

Figure 18.2.3 *(above and at right)* A portion of the synthesis schedule worksheets generated by Pepmaker software for Schedule no. 1 for synthesis of a set of all possible overlapping octamers of sperm whale myoglobin. **(A)** This page of the synthesis schedule is a summary of the features of the protein including its sequence. **(B)** This page of the synthesis schedule shows the sequences of the first 96 peptides that will be synthesized, the first two of which are the controls, PLAQGGGG and GLAQGGGG. Peptide sequences are shown in the conventional amino-to-carboxy-terminal direction (from left to right), with a "<" sign indicating the end attached to the solid phase during synthesis. Because amino acid couplings are carried out in the carboxy-to-amino direction, the first amino acids coupled are at the right-hand end of each sequence, adjacent to the "<".

7. Place the block in a second methanol bath to fully cover the tips. Wash for 2 min. Repeat this washing step again with a fresh methanol bath for a total of three methanol washes.

8. Remove the block and allow it to air dry in an acid-free fume hood for a minimum of 30 min.

   Avoid exposure to acidic fumes as this could prevent efficient coupling in the next step.

   The block can be conveniently left to dry while the amino acid solutions are being dispensed.

Dispense activated amino acid solutions

9. Dispense the required volume of each activated amino acid solution (see Support Protocol 1; 150 µl for gears or 450 µl for macrocrowns) into the appropriate wells of the reaction tray as specified by the synthesis schedule for the coupling cycle (e.g., Fig. 18.2.5).

Perform the amino acid coupling

10. Place the pins in the activated amino acid solutions in the reaction tray, ensuring that the block is correctly oriented before actually lowering the pins into the solution. Incubate ≥2 hr at 20° to 25°C in a polyethylene box with a lid or in a sealable polyethylene bag.

    Coupling begins immediately and is irreversible.

B GENERAL NET SYNTHESIS SCHEDULE NUMBER : 1 Page 2

Amino terminus is printed on the left

1	A 1(1)PLAQGGGG<	49	A 1(5)KHLKTEAE<
2	A 2(1)GLAQGGGG<	50	A 2(5)HLKTEAEM<
3	A 3(1)VLSEGEWQ<	51	A 3(5)LKTEAEMK<
4	A 4(1)LSEGEWQL<	52	A 4(5)KTEAEMKA<
5	A 5(1)SEGEWQLV<	53	A 5(5)TEAEMKAS<
6	A 6(1)EGEWQLVL<	54	A 6(5)EAEMKASE<
7	A 7(1)GEWQLVLH<	55	A 7(5)AEMKASED<
8	A 8(1)EWQLVLHV<	56	A 8(5)EMKASEDL<
9	A 9(1)WQLVLHVW<	57	A 9(5)MKASEDLK<
10	A10(1)QLVLHVWA<	58	A10(5)KASEDLKK<
11	A11(1)LVLHVWAK<	59	A11(5)ASEDLKKH<
12	A12(1)VLHVWAKV<	60	A12(5)SEDLKKHG<
13	A 1(2)LHVWAKVE<	61	A 1(6)EDLKKHGV<
14	A 2(2)HVWAKVEA<	62	A 2(6)DLKKHGVT<
15	A 3(2)VWAKVEAD<	63	A 3(6)LKKHGVTV<
16	A 4(2)WAKVEADV<	64	A 4(6)KKHGVTVL<
17	A 5(2)AKVEADVA<	65	A 5(6)KHGVTVLT<
18	A 6(2)KVEADVAG<	66	A 6(6)HGVTVLTA<
19	A 7(2)VEADVAGH<	67	A 7(6)GVTVLTAL<
20	A 8(2)EADVAGHG<	68	A 8(6)VTVLTALG<
21	A 9(2)ADVAGHGQ<	69	A 9(6)TVLTALGA<
22	A10(2)DVAGHGQD<	70	A10(6)VLTALGAI<
23	A11(2)VAGHGQDI<	71	A11(6)LTALGAIL<
24	A12(2)AGHGQDIL<	72	A12(6)TALGAILK<
25	A 1(3)GHGQDILI<	73	A 1(7)ALGAILKK<
26	A 2(3)HGQDILIR<	74	A 2(7)LGAILKKK<
27	A 3(3)GQDILIRL<	75	A 3(7)GAILKKKG<
28	A 4(3)QDILIRLF<	76	A 4(7)AILKKKGH<
29	A 5(3)DILIRLFK<	77	A 5(7)ILKKKGHH<
30	A 6(3)ILIRLFKS<	78	A 6(7)LKKKGHHE<
31	A 7(3)LIRLFKSH<	79	A 7(7)KKKGHHEA<
32	A 8(3)IRLFKSHP<	80	A 8(7)KKGHHEAE<
33	A 9(3)RLFKSHPE<	81	A 9(7)KGHHEAEL<
34	A10(3)LFKSHPET<	82	A10(7)GHHEAELK<
35	A11(3)FKSHPETL<	83	A11(7)HHEAELKP<
36	A12(3)KSHPETLE<	84	A12(7)HEAELKPL<
37	A 1(4)SHPETLEK<	85	A 1(8)EAELKPLA<
38	A 2(4)HPETLEKF<	86	A 2(8)AELKPLAQ<
39	A 3(4)PETLEKFD<	87	A 3(8)ELKPLAQS<
40	A 4(4)ETLEKFDR<	88	A 4(8)LKPLAQSH<
41	A 5(4)TLEKFDRF<	89	A 5(8)KPLAQSHA<
42	A 6(4)LEKFDRFK<	90	A 6(8)PLAQSHAT<
43	A 7(4)EKFDRFKH<	91	A 7(8)LAQSHATK<
44	A 8(4)KFDRFKHL<	92	A 8(8)AQSHATKH<
45	A 9(4)FDRFKHLK<	93	A 9(8)QSHATKHK<
46	A10(4)DRFKHLKT<	94	A10(8)SHATKHKI<
47	A11(4)RFKHLKTE<	95	A11(8)HATKHKIP<
48	A12(4)FKHLKTEA<	96	A12(8)ATKHKIPI<

Figure 18.2.3 *(continued)*

Wash the pins

11. Remove the block of pins from the amino acid solutions and, if the next cycle is to start immediately, place the block in a methanol bath in which the pins are immersed to half their height (tips are fully immersed). Wash with agitation for 5 min. Flick off excess methanol and allow to air dry for 2 min.

12. Place the block in a DMF bath in which the pins are immersed to half their height (tips are fully immersed). Wash with agitation for 5 min.

    If extra pins are to be added as synthesis progresses (if the peptides being made differ in length), add the pins to the appropriate spaces (see Fig. 18.2.5).

13. Repeat steps 4 through 12 for each cycle of amino acid addition. Follow the synthesis schedule for preparing Fmoc–protected amino acid solutions and for depositing

GENERAL NET SYNTHESIS SCHEDULE NUMBER : 1 Page 3
Bulk solutions for activator and/or additives (150 wells)
Chemistry Group 1 data for synthesis coupling 1
Activator : DIC requires 567.9 mg in 9.0 ml of DMF
Additive 1: HOBt requires 816.4 mg in 35.5 ml of DMF

WEIGHTS FOR INDIVIDUAL AMINO ACID SOLUTIONS

AA	#	Amino acid description	Batch Weight (mg) Target	Actual	DIC (ml)	HOBt (ml)
A	17	Fmoc-L-Ala-OH.H20	..... 155.2	.....	0.94	3.77
D	6	Fmoc-L-Asp(OtBu)-OH	..... 75.1	.....	0.36	1.46
E	12	Fmoc-L-Glu(OtBu)-OH.H2O	..... 150.8	.....	0.68	2.72
F	6	Fmoc-L-Phe-OH	..... 70.7	.....	0.36	1.46
G	14	Fmoc-Gly-OH	..... 116.7	.....	0.79	2.30
H	12	Fmoc-L-His(Boc)-OH.5DCM	..... 176.8	.....	0.68	2.72
I	9	Fmoc-L-Ile-OH	..... 92.3	.....	0.52	2.09
K	19	Fmoc-L-Lys(Boc)-OH	..... 245.4	.....	1.05	4.19
L	17	Fmoc-L-Leu-OH	..... 166.5	.....	0.94	3.77
M	2	Fmoc-L-Met-OH	..... 28.8	.....	0.16	0.62
N	2	Fmoc-L-Asn(Trt)-OH	..... 46.2	.....	0.16	0.62
P	4	Fmoc-L-Pro-OH	..... 43.9	.....	0.26	1.04
Q	5	Fmoc-L-Gln(trt)-OH	..... 95.4	.....	0.31	1.25
R	4	Fmoc-L-Arg(PMC)-OH.3IPE	..... 90.4	.....	0.26	1.04
S	5	Fmoc-L-Ser(tBu)-OH	..... 59.9	.....	0.31	1.25
T	5	Fmoc-L-Thr(tBu)-OH	..... 62.1	.....	0.31	1.25
V	7	Fmoc-L-Val-OH	..... 70.8	.....	0.42	1.67
W	1	Fmoc-L-Trp(Boc)-OH	..... 27.0	.....	0.10	0.41
Y	3	Fmoc-L-Tyr(tBu)-OH	..... 47.7	.....	0.21	0.83

Figure 18.2.4 This page of the synthesis schedule is used for the preparation of activated amino acid solutions. It shows the amounts of each amino acid (represented by the single letter code, A through Y, along the left-hand margin), activator (diisopropylcarbodiimide [DIC] in dimethylformamide [DMF]), and catalyst (additive; 1-hydroxybenzotriazole [HOBt] in DMF) needed for the first amino acid coupling cycle. (In this example the amounts are calculated for a 250-µl reaction volume.) The amino acid powder is dissolved in the HOBt/DMF solution (right-hand column) before activation with DIC/DMF solution.

```
GENERAL NET SYNTHESIS SCHEDULE NUMBER : 1          Page 4
        PIN POSITIONS for Synthesis coupling  1
                        NEW PIN POSITIONS
A 1(1) TO B 6(5)

                Well positions for amino acid dispensing

A  A10(1)  A 2(2)  A 5(2)  A12(4)  A 4(5)  A 6(6)  A 9(6)
     A 7(7)  A 1(8)  A 5(8)  B11(1)  B 2(3)  B 4(3)  B 7(3)
     B11(3)  B 8(4) TO B 9(4)

D  A 3(2)  A10(2)  A 3(4)  A 7(5)  B 3(3)  B 6(4)

E  A 1(2)  A 9(3)  A12(3)  A11(4)  A 1(5)  A 6(5)  A 6(7)
     A 8(7)  B 6(1)  B10(1)  B 1(4)  B 1(5)

F  A 4(3)  A 2(4)  A 5(4)  B 7(1)  B12(2)  B 3(4)

G  A 1(1) TO A 2(1) A 6(2)  A 8(2)  A12(5)  A 8(6)  A 3(7)
     B 1(1) TO B 2(1) B10(2)  B 1(3)  B 6(3)  B 3(5)  B 6(5)

H  A 7(1)  A 7(2)  A 7(3)  A 7(4)  A11(5)  A 4(7) TO A 5(7)
     A 4(8)  A 8(8)  B 2(2)  B 5(2)  B 8(2)

I  A11(2)  A 1(3)  A10(6)  A10(8)  A12(8)  B 8(1)  B12(1)
    TO B 1(2) B 7(4)

K  A11(1)  A 5(3)  A 1(4)  A 6(4)  A 9(4)  A 3(5)  A 9(5)
    TO A10(5) A12(6) TO A 2(7) A10(7)  A 7(8)  A 9(8)  B 3(1)
     B10(3)  B 5(4)  B10(4)  B12(4)

L  A 4(1)  A 6(1)  A12(2)  A 3(3)  A11(3)  A 8(4)  A 8(5)
     A 4(6)  A 7(6)  A11(6)  A 9(7)  A12(7)  B 5(1)  B 4(2)
     B12(3)  B 2(4)  B 2(5)

M  A 2(5)  B 8(3)

N  B11(2)  B 9(3)

P  A 8(3)  A11(7)  A11(8)  B 9(2)

Q  A 3(1)  A 9(2)  A 2(8)  B 5(3)  B 5(5)

R  A 2(3)  A 4(4)  B 7(2)  B 4(4)

S  A 6(3)  A 5(5)  A 3(8)  B 9(1)  B 6(2)

T  A10(3)  A10(4)  A 2(6)  A 5(6)  A 6(8)

V  A 5(1)  A 8(1)  A12(1)  A 4(2)  A 1(6)  A 3(6)  B 3(2)

W  A 9(1)

Y  B 4(1)  B11(4)  B 4(5)
```

Figure 18.2.5 This page of the synthesis schedule identifies which wells of the reaction tray receive which activated amino acid solution. Each amino acid solution is identified along the left-hand margin using the single-letter amino acid code. Individual reaction trays and pin holders (A or B in this case) are identified by letters of the alphabet, and the paired numbers—e.g., 10(1) for well 10H—identify individual wells within reaction trays according to the numbering system illustrated in Figure 18.2.6.

aliquots of the activated amino acids to the appropriate wells of the reaction tray for each cycle.

Two coupling cycles can be carried out during a normal working day, and a third coupling can be carried out overnight, so a total of three amino acids can be added to each pin during a 24-hr period.

14. Deprotect the final Fmoc amino acid by repeating steps 4 through 8. Then proceed with step 15 or step 16.

15. *Optional:* For B cell epitope scanning or for T helper cell epitope scanning, the N-terminus of the peptide can be capped by acetylation (see Support Protocol 2). To allow later recapture onto avidin, the N-terminus of the peptide can be capped with biotin or long-chain biotin (see Support Protocol 3).

Deprotect the side chains

16. Dispense the required volume of SCD solution into a bath or tubes (1.5 ml/tube) and fully immerse the peptide-bearing portion of the pins. Cover bath or cap tubes and incubate 2.5 hr at 20° to 25°C.

CAUTION: SCD solution is a toxic, corrosive liquid.

For blocks where the peptide is to remain attached to the pin during side chain deprotection (i.e., NCP, GAP, and DKP kits), side chain deprotection is carried out in a bath of reagent. For pins where the peptide is simultaneously side chain deprotected and cleaved from the pin (MPS kits), the process is carried out in individual 10-ml capped conical polypropylene centrifuge tubes that become the containers for the recovered peptide.

17a. *For NCP, GAP, or DKP kits:* Wash the pins 3 times in acidified methanol to remove the SCD solution prior to further treatment.

17b. *For MPS kit:* In a good chemical fume hood, reduce the volume of SCD solution containing cleaved peptide to ~0.1 ml either with a gentle stream of dry nitrogen gas or in a centrifugal vacuum drier (e.g., Speedvac) equipped to handle corrosive fumes. Precipitate the peptide in the remaining solution with 8 ml of 1:2:0.003 ether/petroleum ether/2-ME. Decant and discard the supernatant, and wash the

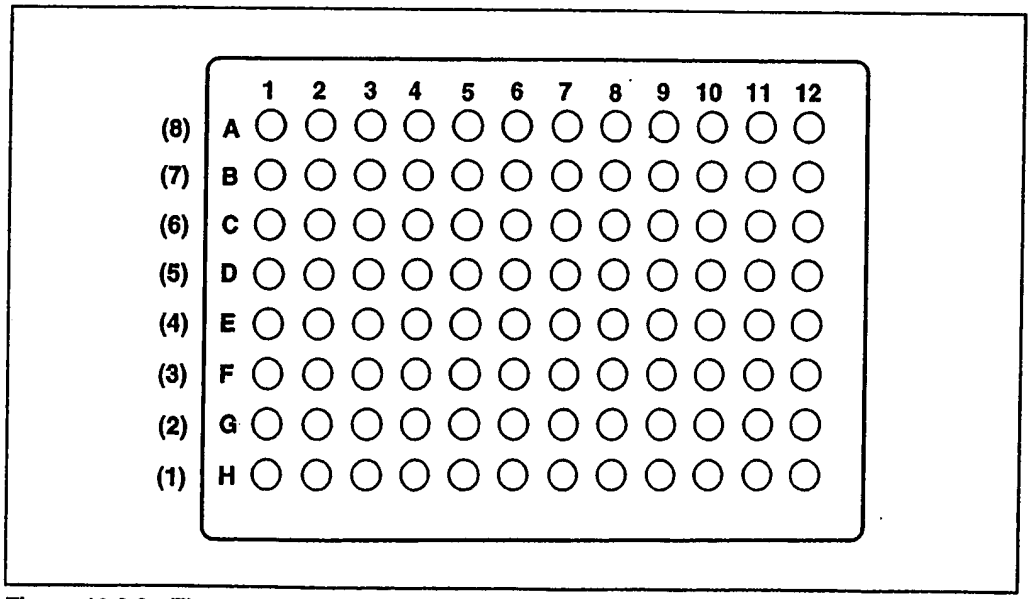

Figure 18.2.6 The numbering system for pins and reaction trays for Chiron Technologies' multipin synthesis system. Each well is identified by a pair of numbers rather than a number and a letter, e.g, 1(1). The first number identifies the column number; the second (in parentheses) identifies the lettered row, beginning with H as (1) and ending with A as (8).

precipitated peptide with 4 ml of 1:2 ether/petroleum ether. Dry the precipitate with a gentle stream of dry nitrogen.

CAUTION: Ether/petroleum ether/2-ME and ether/petroleum ether solutions are highly flammable.

These dry peptides can now be redissolved for assay purposes or may be further purified, e.g., by HPLC (see UNIT 11.6).

Prepare the peptides

18a. *For GAP kits:* Add 0.7 ml of 0.1 M NaOH to each tube of a rack of 96 1-ml polypropylene tubes. Place the pins into the solution and incubate ~1.5 hr to cleave the peptides from the pins. Immediately after cleavage, neutralize with one equivalent of 0.1 M acetic acid.

Alternatively, the 0.1 M NaOH solution can contain 40% (v/v) acetonitrile to facilitate solubilization of the more hydrophobic peptides.

The incubation time can be shorter if the tubes are sonicated during cleavage.

18b. *For DKP kits:* Cleave the peptide from the pin in a suitable, reasonably well-buffered solution with a pH >7 overnight (16 hr).

The solution for cleavage—e.g., 0.1 M sodium phosphate, pH 8, or 0.1 M HEPES, pH 8—can be chosen to be compatible with the assay for which the peptides will be used; the solution should have a buffering capacity of ~0.05 M.

Again, an organic modifier such as acetonitrile can be added to the cleavage solution to facilitate solubilization of the more hydrophobic peptides.

18c. *For NCP kits:* Prepare the pins for binding assays by floating the block, pin-side down, in sonication buffer and sonicating 10 min at ~60°C. Rinse the pins first in water, then in 20° to 45°C methanol for immediate use in an assay or air dry the pins for storage until they are used in an assay.

PREPARING ACTIVATED Fmoc–PROTECTED AMINO ACID SOLUTIONS

SUPPORT PROTOCOL 1

Activated 9-fluorenylmethyloxycarbonyl (Fmoc)–protected amino acid solutions are prepared in two steps. First, the protected amino acid is dissolved in a solution of the catalyst, 1-hydroxybenzotriazole (HOBt) in dimethylformamide (DMF). Then, just before dispensing, the amino acid is activated by adding an activating agent. The following procedure illustrates the use of diisopropylcarbodiimide (DIC) as the activating reagent. DIC is a liquid and can be measured by volume.

Additional Materials (also see Basic Protocol)

9-fluorenylmethyloxycarbonyl (Fmoc)–protected amino acids with side-chain-protecting groups (Sigma, Bachem, Novachem, or Chiron Technologies), stored at 4°C
Catalyst: 1-hydroxybenzotriazole (HOBt)
Activating agent: diisopropylcarbodiimide (DIC)
Dimethylformamide (DMF), amine-free
Ethanol, analytical reagent grade
5- or 10-ml glass, polyethylene, or polypropylene bottles with inert (e.g., polythylene, Teflon) lids and liners

1. Remove the Fmoc–protected amino acids from the refrigerator and allow them to come to room temperature before weighing them out.

Warming the containers to room temperature avoids the possibility of uptake of moisture from the air onto the cold solids.

The side chains of the amino acids must also be protected during peptide synthesis: t-butyl ether is used for serine, threonine, and tyrosine; t-butyl ester is used for aspartic acid and glutamic acid; t-butoxycarbonyl (t-Boc) is used for lysine, histidine, and tryptophan; 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) is used for arginine; and trityl (Trt) is used for cysteine. If benzotriazolyl-N-oxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) or benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) activation is to be used, trityl protection should be used for asparagine and glutamine. BOP and PyBOP require greater care in handling and different protection should be used for some amino acids.

2. Weigh individual amino acids and HOBt using the quantities specified for the current synthesis cycle (e.g., see Fig. 18.2.4) into separate appropriately sized clean and dry glass, polyethylene, or polypropylene bottles. Rinse the spatula with ethanol and dry it after weighing each reagent.

   The bottle caps and inserts must be inert to any of the reagents or solvents used in making up the activated solutions (they must be Teflon, not rubber).

   Care should be taken to avoid cross-contamination of amino acids by rinsing the spatula in ethanol between weighings (the spatula must be dry before each use) and by making sure that all lids, as well as the containers, are labeled so they can be replaced on the correct bottles after weighing has been completed.

3. Measure the appropriate amount of DIC (see "Activator" as in Fig. 18.2.4).

   DIC is a liquid so it is more convenient to measure it by volume rather than by weight. Multiply the indicated weight by 1.23 (based on the density of DIC, 0.815 g/ml) as a conversion factor from the calculated weight to get the required volume of DIC in microliters.

4. Prepare HOBt and DIC solutions, by pipetting the appropriate volumes of purified (amine-free) DMF as shown on the synthesis schedule (e.g., see Fig. 18.2.4).

   Both reagents should be fully dissolved in the DMF before using them to prepare the activated amino acid solutions.

5. Add the specified volume of HOBt/DMF solution (e.g., Fig. 18.2.4, column headed "HOBt") to the individual amino acids.

   Make sure the amino acids are completely dissolved before adding the activator solution.

   Unactivated amino acid solutions may be stored a few days at 4°C.

6. Activate the individual amino acid solutions by adding the specified volume of DIC/DMF solution to each amino acid solution (e.g., Fig. 18.2.4, column headed "DIC"). Mix thoroughly and use immediately for peptide synthesis.

   Activated amino acids should be prepared immediately before use and any excess should be discarded.

SUPPORT PROTOCOL 2

N-TERMINAL ACETYLATION OF PEPTIDES

N-terminal capping of the peptides is carried out after a final 9-fluorenylmethyloxycarbonyl (Fmoc)–deprotection cycle and prior to side chain deprotection. The process is similar to coupling of amino acids, except that in the case of acetylation the active reagent can be acetic anhydride rather than acetic acid. Acetic anhydride does not require activation. If acetic anhydride is not available, simply use acetic acid as if it were an amino acid (see Support Protocol 3).

Additional Materials (also see Basic Protocol)

Acetylation solution (see recipe), prepared just before use
Pins with completed peptides (see Basic Protocol)

1. Add freshly prepared acetylation solution to appropriate bath container.
2. Immerse the pins with completed peptides in acetylation solution and incubate 90 min at room temperature.
3. Wash the pins in a methanol bath and air dry.

The pins can now be used for side chain deprotection (Basic Protocol, step 16).

N-TERMINAL BIOTINYLATION OF PEPTIDES

SUPPORT PROTOCOL 3

Biotin can also be coupled to the N-terminus of peptides after N-terminal deprotection and before side chain deprotection. The reagent is used as if it were an amino acid, using the same solvent, activating agent, and catalyst.

Additional Materials (also see Basic Protocol)

Biotin or long-chain biotin
Dimethylformamide (DMF), amine-free
Diisopropylcarbodiimide (DIC)
Pins with completed peptides (see Basic Protocol)

1. Dissolve biotin in amine-free DMF to a concentration of 125 mM.
2. Prepare a 10× solution of DIC (activating agent) by dissolving 158 mg DIC in 1 ml DMF and prepare a 10× solution of HOBt (catalyst) by dissolving 192 mg HOBt in 1 ml DMF.
3. Activate the biotin with the 10× concentrate solutions of activation agent and catalyst (80:10:10 [v/v/v]).
4. Dispense 150 µl/well (for gears) or 450 µl/well (for macrocrowns) into reaction trays.
5. Immerse the pins with completed peptides in the reaction tray and incubate ≥2 hr.
6. Wash the pins in methanol.

The pins can now be used for side chain deprotection (see Basic Protocol, step 16).

REAGENTS AND SOLUTIONS

Use Milli-Q water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

Acetylation solution

For 200 ml:
193 ml dimethylformamide (DMF)
6 ml acetic anhydride
1 ml *N*-ethyldiisopropylamine
Prepare immediately before use and discard after use

DO NOT expose pins to acetic anhydride at any other time except during acetylation. Also, do not store acetic anhydride anywhere near where peptide synthesis is performed.

The DMF does not need to be amine-free.

20% piperidine/DMF

Prepare a 20% (v/v) solution of the best quality piperidine available in analytical reagent–grade dimethylformamide (DMF). Prepare a fresh solution for each synthesis (solution can be reused several times within a synthesis). Store at room temperature in an amber bottle containing activated molecular sieves to remove moisture.

CAUTION: This solution is highly flammable and toxic.

continued

If high-quality piperidine is not available, it may have to be treated with solid sodium hydroxide and redistilled.

DMF need not be amine-free.

Side chain deprotecting (SCD) solution

33 parts (v/v) trifluoroacetic acid
1 part (v/v) ethanedithiol
2 parts (v/v) anisole
2 parts (v/v) thioanisole
2 parts (v/v) H_2O
Prepare immediately before use and do not store or reuse

CAUTION: This solution is corrosive and extremely malodorous. Contamination of the laboratory, especially with ethanedithiol, should be avoided. Wipe the outside of ethanedithiol-contaminated equipment or containers with dilute, 0.1% aqueous hydrogen peroxide to oxidize ethanedithiol to a nonodorous compound before removing the container from the fume hood. DO NOT allow hydrogen peroxide to contact other readily oxidizable materials or reagents.

Sonication buffer

1% (w/v) SDS
0.1 M sodium phosphate buffer, pH 7.2 (*APPENDIX 2E*)
0.1% (v/v) 2-mercaptoethanol (2-ME)
Store at room temperature up to 1 week

CAUTION: Before discarding sonication buffer, destroy remaining 2-ME by adding 2 ml 30% hydrogen peroxide per liter of buffer.

COMMENTARY

Background Information

The multipin method was developed by Dr. H.M. Geysen and coworkers (Geysen et al., 1984, 1987) as a scanning method for linear antibody-defined epitopes. Eventually in the late 1980s, the method was adapted to parallel synthesis of cleaved (soluble) peptides (Maeji et al., 1990), opening the way for systematic scanning of T helper (Reece et al., 1993) and cytotoxic epitopes (Burrows et al., 1994). Initially only suitable for synthesis of short peptides (up to 10 amino acid residues), the method can now routinely produce peptides of up to 20 residues of acceptable quality for initial screening experiments (Valerio et al., 1993).

Critical Parameters

Successful peptide synthesis requires reagents of a quality appropriate to the particular step, and the careful application of those reagents. For example, the protected amino acids need to be free of reactive counterions such as dicyclohexylamine (DCHA), contaminating unprotected amino acid, isomers such as the D-amino acid, and water. Check carefully that the amino acid as supplied is EXACTLY the same as specified in the manual or on the software. Apart from quality testing each amino acid, the best assurance of quality is to buy only from reputable suppliers.

Dimethylformamide (DMF) is the primary solvent for carrying out reactions (couplings) on pins. Its low volatility and moderate polarity make it suitable for dissolving the amino acids and solvating the graft polymer/growing peptide on the pin surface. Purity is not critical for some (washing) steps, but is critical for the DMF used just before and during amino acid coupling. Presence of excessive amine in the DMF results in loss of activated amino acid because the amino acid couples to the amine rather than to the peptide on the pin. Fortunately, the pin system allows use of substantial molar excesses of incoming amino acid (typically 6- to 1000-fold), so loss of some amino acid is not disastrous. Fresh DMF of the best available grade should be used for the coupling, and it is recommended that the amine level be tested using the FDNB test (Stewart and Young, 1984).

Liberal use is made of methanol as a washing solvent. Analytical reagent grade methanol is readily available at low cost in large containers (20 or 200 liters) and is relatively easy to dispose of. It is possible to reduce the use of methanol by reusing it for washes: the last wash

bath in any series should be in fresh (pure) methanol. In the next round of washes, the former last bath is then reassigned as the second-to-last wash, the previously second-to-last bath becomes the third-to-last, and so on. For each synthesis cycle, the first wash bath in the series is the one which is discarded. The presence of methanol is undesirable during reactions on the pins, but as it evaporates readily it can be easily removed by standing the block in a moving stream of air, such as the opening of an operating chemical fume hood. Methanol will dry more rapidly and the methanol-washed pins will take up less moisture from the air if the methanol is warm (e.g., prewarmed to 45°C in a closed bottle in a water bath).

Other solvents (e.g., ether, petroleum ether, acetonitrile) should be the best available grade.

Carrying out the correct synthesis of the peptides requires that all steps are performed with a very high level of attention to detail. All cyclically repeated steps (washes and deprotections) must be performed, and the activation and dispensing of the amino acids for each coupling cycle must be carried out exactly, or the peptides made may have the incorrect sequence, may be missing an amino acid, or may be truncated. Computerized equipment is available for assisting with the accurate dispensing of amino acids to the wells in a reaction tray (e.g., "Pin-Aid," Chiron Technologies; Carter et al., 1992). The growing peptides must not be subjected to conditions that would prematurely block or deprotect the side chains (for example, from premature exposure to acetic anhydride or trifluoroacetic acid, which should be stored well away from where peptide synthesis is being performed).

As a spot test for correct completion of all the steps of synthesis, it is wise to synthesize controls on each block of 96 pins. For noncleavable peptides, these controls can be peptide sequences that can be probed with an antibody known to react with the peptide. In this case, one of the two peptides should be a negative control, such as a randomized sequence. For cleavable peptides, the quantity and quality of the controls can be monitored by the usual techniques of HPLC (*UNIT 11.6*), amino acid analysis (*UNIT 11.9*), and mass spectrometry (Chapter 16). Ultimately, proof that an assay result is a function of the particular peptide made has to rely on a confirmatory experiment carried out with more highly-characterized peptide or on analysis of a sample of the particular peptide used in the experiment.

Once peptides have been made, they need to be handled and stored carefully to prevent degradation. Noncleavable peptides (pins) should be stored dry in a refrigerator after removal of any bound protein. If stored with desiccant they should be stable for months to years. Cleaved peptides can be stored frozen or as dry powder. After a long period of storage, it is wise to reassay controls or confirm the quality of the stored peptide by analysis.

Another parameter critical to data from large numbers of peptides is to ensure that the identity of each peptide is properly tracked and that activity is not ascribed to the wrong peptide. Consistent use of the 8 × 12 microtiter plate format for synthesis, storage, assay, and use of computerized records for tracking all three processes can help avoid mistakes. Tracking and control is particularly easy if the assay data is read directly from a microtiter plate reader to a computer that is programmed with the peptide information because this method avoids manual data transcription.

Anticipated Results

For a noncleavable pin-peptide synthesis, two control peptides, one of which is reactive with a monoclonal antibody in ELISA and the other serving as a nonbinding peptide control, should show the specific binding expected based on past data. For cleaved peptides, the yield of control peptide should be in the range expected from the stated pin loading (substitution level), e.g., 1 μmol for GAP and DKP kits or 5 μmol for the MPS kit. Purity of the cleaved controls should be consistent with the results of previous batches and should be of an acceptable standard.

Testing of a systematic set of peptides in a bioassay can give data that is interpretable without recourse to additional controls, because a systematic set of peptides through a protein includes many sequences that are unlikely to be reactive sequences, i.e., they act as internal negative controls. Figure 18.2.7 shows one set of ELISA data from scanning noncleaved peptides with a monoclonal antibody. In screening for T helper cell responsiveness it is critical to include many control cultures, not only controls with no peptide added but also controls with nonstimulatory peptide. Systematic sets of peptides automatically include such controls (Reece et al., 1994).

Time Considerations

If amino acid coupling is carried out at 3 cycles/day, which can fit into a conventional working day, then it will take up to 2 weeks to make a set of 15-mers, as there is extra time required for side chain deprotection and drying down (depending on the peptide format). Although this may seem slow, the fact that hundreds or thousands of peptides can be made simultaneously means that a project requiring large numbers of peptides is completed in a very short time. Indeed, the rate-limiting step may be the time it takes to carry out the assays on the large number of peptides when they become available.

From this perspective, biotinylated peptides produced on glycine acid peptide (GAP), diketopiperazine (DKP), or multiple peptide syn-

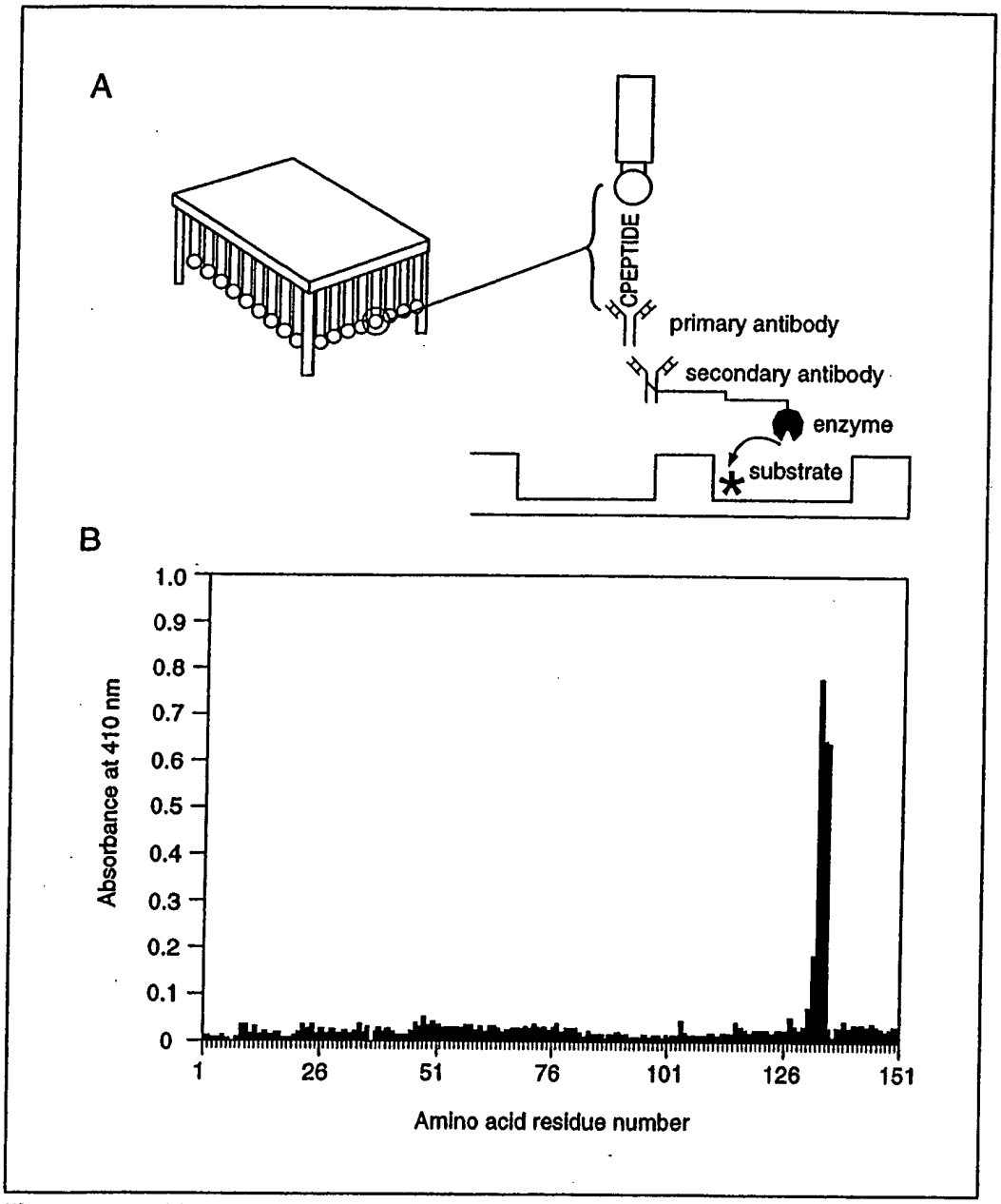

Figure 18.2.7 Multipin capture ELISA. (**A**) Setup for multipin capture ELISA. Pins (gears) with peptides covalently attached are incubated in primary antibody, secondary antibody, and substrate developer in ELISA plates. The absorbance is measured and the resulting absorbance values are graphed versus peptide number, corresponding to the N-terminal residue number of the peptide in the protein sequence. (**B**) Peptide pin capture ELISA results with a monoclonal antibody against pins bearing octamer peptides of gonococcal pilin protein. All the peptides that show high readings contain a significant portion of the epitope. (Diagram courtesy of Dr. Fred Cassels, Walter Reed Army Institute of Research, Washington, D.C.)

thesis (MPS) pins have a great advantage over the noncleavable peptide (NCP) pin-bound peptides, as the latter can only be assayed once a day, whereas hundreds of parallel assays can be carried out on all biotinylated peptides at once. Reading data directly into a computer enables the massive amounts of data to be stored efficiently for later analysis.

Dispensing amino acids can be carried out efficiently by two people, one reading out the position into which the amino acid is to be dispensed and the other doing the actual dispensing. The passive partner (reader) can also act as a cross-checker to ensure no mistakes are made. If a computer-controlled pointing device is used, accuracy is improved and dispensing becomes a one-person operation. For large syntheses (>200 peptides), it is important that the dispensing be fast and accurate so that three couplings can be carried out per day.

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Contributed by Stuart J. Rodda
Chiron Technologies Pty. Ltd.
Victoria, Australia